The role of Protein Phosphatase 2A (PP2A) in myocardial ischaemia/reperfusion injury

by
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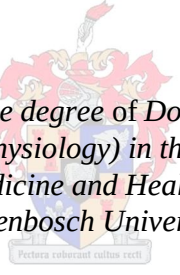

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Declaration

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ABSTRACT

Ischaemic heart disease is a major contributor to global morbidity and mortality rates. Manoeuvres such as ischaemic preconditioning confer cardioprotection against ischaemia/reperfusion (I/R) injury by activating several intracellular signalling pathways. These pathways have been defined solely in terms of the kinases involved, despite the realization in recent years that protein phosphatase activity also contributes significantly to the attributes of the propagated signal. Protein phosphatase 2A (PP2A) is a heteromultimeric enzyme involved in an array of phosphatase reactions. We hypothesized that PP2A is an important participant in the myocardial response to I/R by regulating intracellular signalling.

This project aimed to (i) characterize PP2A during myocardial I/R; (ii) determine the importance of its contribution to the cellular response to I/R; and (iii) investigate its role in the signalling pathways mediated by PKB/Akt, GSK-3β, ERK p42/p44 and p38 MAPK.

Two models were used to characterize PP2A during I/R: (i) H9c2 cells exposed to simulated ischaemia (SI) buffer in conjunction with hypoxia (0.5% O_2) for a maximum of 2 hours, followed by reoxygenation in standard growth medium for up to 30 minutes; and (ii) isolated working rat hearts exposed to a maximum of 20 minutes global ischaemia and 10 minutes reperfusion. In both models samples were collected at several time points during I/R for Western blotting analysis. PP2A-C (the catalytic subunit) accumulated in the nucleus during early ischaemia, but later redistributed to the cytosol. At the end of ischaemia there was an elevation of PP2A-C relative to PP2A-A in the unfractionated whole cell preparation concomitant with an increase in the inhibitory phosphorylation of PP2A-C.

The impact of PP2A activity was evaluated by either inhibiting PP2A using okadaic acid (OA, 10 nM) or activating it by administering FTY720 (1 μM) in an isolated working rat heart model exposed to either 35 minutes of regional ischaemia (RI) with infarct size (IFS) as primary end-point, or 20 minutes global ischaemia (GI) with functional recovery as end-point. The results showed that the pre-ischaemic administration of OA or FTY720 reduced or exacerbated IFS respectively, indicating that PP2A activation during I/R favours cell death.

OA and FTY720 were also employed to assess the contribution of PP2A to intracellular signalling in an isolated working rat heart exposed to I/R. Samples were collected at several timepoints and analyzed using Western Blotting. Pre-ischaemic administration of OA enhanced the phosphorylation of PKB/Akt, ERK p42/p44 and GSK-3β at the onset of reperfusion, while FTY720 given before ischaemia reduced the phosphorylation of GSK-3β, p38 MAPK and PKB/Akt at the end of ischaemia and onset of reperfusion.

In summary, PP2A is part of an early nuclear-based response to ischaemia, while long-term ischaemia induces an increase in PP2A-C. A portion of this PP2A-C is stored in an inactive form, while an active portion acts as a regulator of the pro-survival signalling components PKB/Akt, GSK-3β and ERK p42/p44 at the end of ischaemia and the onset of reperfusion. PP2A is therefore an important component of the myocardial response to I/R by regulating pro-survival signalling.

OPSOMMING

Iskemiese hartsiekte is een van die belangrikste komponente wat bydra tot globale morbiditeit en mortaliteit. Ingrepe soos iskemiese prekondisionering aktiveer veelvoudige intrasellulêre seintransduksiepaaie om kardiobeskerming teen iskemie/herperfusie (I/H)-besering te ontlok. Die kinases betrokke in hierdie seintransduksiepaaie is reeds deeglik nagevors, terwyl die potensiële belang van die proteïenfosfatases in seintransduksie tot onlangs misken is. Ons hipotese was dat Proteïenfosfatase 2A (PP2A), wat in 'n wye verskeidenheid fosfatase reaksies betrokke is, 'n belangrike rolspeler in die miokardiale reaksie op I/H-besering is, deur deelname aan die regulering van intrasellulêre seintransduksie.

Hierdie projek het ten doel gehad om (i) PP2A te karakteriseer tydens miokardiale I/H; (ii) die belang van PP2A in die sellulêre reaksie op I/H-besering te bepaal; en (iii) PP2A se rol in die seintransduksiepaaie, gemedieer deur PKB/Akt, GSK-3β, ERK p42/p44 en p38 MAPK, te evalueer.

Twee modelle is aangewend om PP2A tydens I/H te karakteriseer: (i) H9c2-selle blootgestel aan 'n simuleerde iskemiebuffer tesame met hipoksie (0.5% O_2) vir 'n maksimum van 2 uur gevolg deur heroksiginasie in standaardgroeimedium vir verskillende tydsperiodes tot 'n maksimum van 30 minute; en (ii) geïsoleerde, werkende rotharte blootgestel aan 'n maksimum van 20 minute globale iskemie en 10 minute herperfusie. In beide modelle is monsters op verskillende tye versamel vir Western-kladanalise. Tydens vroeë iskemie het PP2A-C in die kern toegeneem, waarna dit met verloop van tyd na die sitosol herversprei het. Teen die einde van iskemie was daar 'n toename in die vlakke van PP2A-C relatief tot PP2A-A in ongefraksioneerde weefselhomogenate, tesame met 'n toename in die inhibitoriese fosforilering van PP2A-C.

Die belang van PP2A-aktiwiteit is ondersoek deur die effek te bepaal van die inhibisie of aktivering daarvan op infarktgrootte (IFS) en funksionele herstel in 'n geïsoleerde werkende rothartmodel, blootgestel aan onderskeidelik 35 minute streeksiskemie (RI) of 20 minute globale iskemie. Pre-iskemiese toediening van die PP2A-inhibitor okadaïensuur (OA, 10 nM), of aktiveerder FTY720 (1 µm) het infarktgrootte respektiewelik beperk of vergroot. PP2A-aktivering tydens I/H is dus nadelig.

OA en FTY720 is ook aangewend om die bydrae van PP2A tot I/H-verwante, intrasellulêre seintransduksie in die geïsoleerde, werkende rothart te bepaal. Monsters is op verskeie tydintervalle versamel en ontleed deur gebruik te maak van die Western-kladtegniek. Pre-iskemiese toediening van OA het die fosforilering van PKB/Akt, ERK p42/p44 en GSK-3β by die aanvang van herperfusie bevoordeel, terwyl pre-iskemiese toediening van FTY720, die

fosforilering van GSK-3β, p38 MAPK en PKB/Akt aan die einde van iskemie en die begin van herperfusie verminder het.

Ter opsomming: PP2A is deel van ‘n vroeë gelokaliseerde kerngebaseerde reaksie op iskemie, terwyl langdurige iskemie ‘n toename in PP2A-C relatief tot PP2A-A induseer. ‘n Deel van hierdie PP2A-C is onaktief, terwyl die res funksioneer in die regulering van die seintransduksiekomponente PKB/Akt, GSK-3β en ERK p42/p44 wat oorlewing fasiliteer met die aanvang van herperfusie. PP2A is dus ‘n belangrike komponent in die miokardiale reaksie op I/H deurdat dit tot die beheer van seintransduksiepaaie bydra.

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"...all that we have accomplished You have done for us."

Isaiah 26:12
The Bible

"I want to set the story straight, it's time to tell the truth. I never could have come so far, if it were not for You"

All because
Tree63
The life and times of the absolute truth, Lush Records, 2002.

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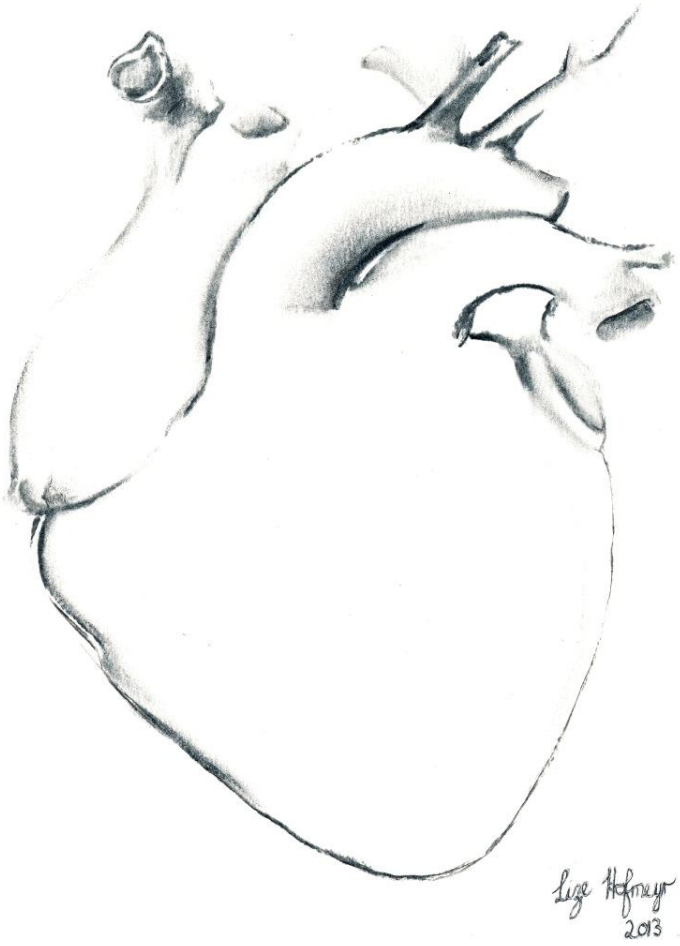
CHAPTER 1

Introduction

"I praise you because I am fearfully and wonderfully made; your works are wonderful, I know that full well."

Ps. 139:14
The Bible



Chapter 1 - Introduction

The ischaemic heart

Ischaemic heart disease

A recent report by the World Health Organisation (WHO, Global status report on noncommunicable diseases 2010) highlighted a shift in the global burden of disease from more traditional communicable causes of morbidity and mortality to non-communicable diseases (NCDs): in 2008 63% of deaths globally were due to NCDs. Non-communicable diseases include conditions such as obesity, hypertension, diabetes and cardiovascular disease – all conditions which are intrinsically linked to life-style. Traditionally these health issues were confined to the affluent nations of the world, but trends reveal that low- and middle-income countries are also increasingly being exposed to these diseases as life-styles shift and westernize, while populations age. In fact 80% of deaths associated with NCDs were reported in low- and middle-income countries (WHO, 2010). Amongst these NCDs one of the primary causes of mortality is ischaemic heart disease (IHD).

In 1997 Murray and Lopez published a study in which they projected that ischaemic heart disease would remain the top cause of death from 1990 through to 2020, as well as be in the top 5 causes of disability adjusted life years (DALYs) in both developed and developing regions. Nine years later Mathers and Loncar (2006) published updated and extended projections of global morbidity and mortality to the year 2030. The trend however remains the same: in high-, middle- and low-income countries ischaemic heart disease is expected to remain one of the top 5 causes of death. Assessing the situation in 2010, Lozano *et al.* confirmed the WHO-report, as well as these projections: From 1990 to 2010 the number of global deaths attributable to NCDs increased by approximately 8 million deaths per year, an increase of 30%; largely due to increases in population size and age. Ischaemic heart disease caused the largest fraction of deaths in 2010 at 13.3%. Almost unbelievably, stroke and IHD combined contributed to a quarter of global deaths, an increase from a fifth 20 years ago.

Ischaemic heart disease is therefore clearly a health concern of global proportions. As the world population increases and grows older and unhealthy life-styles persist, the impact of ischaemic heart disease will also increase. Since this is not a new problem, and in fact it has its genesis in the resource-rich nations, the question arises: What is the current approach to treating and managing the ischaemic heart?

Treating and managing the ischaemic heart

In the real-world clinical setting there are at least three situations which are associated with myocardial ischaemia: (1.) heart transplantation; (2.) cardiac surgery in conjunction with cardiopulmonary bypass; and (3.) myocardial infarction (MI). Of these three, myocardial infarction has probably received the most attention from researchers, since it presents an unpredictable, medical emergency with potentially fatal consequences. We will therefore primarily focus on this setting.

Myocardial ischaemia is defined as insufficient blood supply to the heart (Stedman's Medical Dictionary). This has two fundamental consequences: (1.) There is an inadequate supply of oxygen and nutrients to meet the heart's high metabolic demands; and (2.) Metabolic waste products cannot be drained away from the heart, leading to their toxic accumulation. Sustained ischaemia is therefore associated with cell death (Reimer *et al.*, 1977; Jennings & Reimer, 1983).

There are several cellular mechanisms underlying the injury inflicted by ischaemia. The cessation of blood flow to the heart almost immediately places its energy metabolism under pressure. In the absence of sufficient oxygen levels oxygen-dependent mitochondrial metabolism diminishes. Within the first seconds of ischaemia, however there is an acute, transient increase in anaerobic glycolytic rate (approximately 60-90 seconds), whereafter the rate of glycolysis slows down. Creatine phosphate reserves are also rapidly consumed. The degree and severity of ischaemia will determine how long glycolysis continues (Jennings *et al.* (1990) reported 40-60 minutes), but after some time it too will finally grind to a halt whilst adenosine triphosphate (ATP) levels are spent. This severely compromises the cell's ATP-dependent capacity to maintain normal trans-membrane ion gradients (Jennings *et al.*, 1990; Meisenberg & Simmons, 1998; Gurbel *et al.*, 1998; Opie, 2004).

ATP-dependent proteasomal processes of the ubiquitin-proteasome system (UPS) will also be inactivated, thereby reducing the cardiomyocyte's ability to maintain protein homeostasis and leading to an accumulation of misfolded and damaged proteins and protein aggregates, which in turn will induce proteotoxic and endoplasmic reticulum (ER) stress (Churchill *et al.*, 2010; Divald *et al.*, 2010; Calise & Powell, 2013). ER stress refers to the build-up of misfolded and damaged proteins inside the ER and, if allowed to progress, will eventually stimulate apoptosis (Wang *et al.*, 2008). This ischaemia-induced loss of UPS function is however seemingly compensated for by an increase in autophagy inside the cell (Yan *et al.*, 2005; Dosenko *et al.*, 2006; Zheng *et al.*, 2011; Calise & Powell, 2013). Autophagy, specifically macroautophagy, is the process by which double-membrane vacuoles are formed around proteins, protein aggregates and even whole organelles in order to remove these cytosolic components through the merger of these vacuoles (autophagosomes) with lysosomes, thereby degrading and effectively recycling the

autophagosomal content (Nishida *et al.*, 2009; Dong *et al.*, 2010). It is clear from this definition that autophagy entails more than simply protein homeostasis, we will however for simplicity focus on autophagy in only this context. This process seems to be an adaptive response as it removes dysfunctional proteins, as well as compromised mitochondria and supplies substrates for energy metabolism which rival the oxygen efficiency of glucose (Gottlieb *et al.*, 2009; Dong *et al.*, 2010; Loos *et al.*, 2011). Despite these beneficial effects, excess autophagy can also in itself lead to cell death (Valentim *et al.*, 2006; Matsui *et al.*, 2008; Nishida *et al.*, 2009). Autophagy is however also an ATP-dependent process which will not be sustained if the ischaemic insult is prolonged and severe (Loos *et al.*, 2011).

In conjunction with the depletion of energy resources, there is an accumulation of metabolic waste-products such as lactate, creatine, inorganic phosphates, hydrogen ions (H^+), etc. This leads to a detrimental reduction in the intracellular pH, as well as an increase in the intracellular osmolarity (Jennings *et al.*, 1990; Meisenberg & Simmons, 1998; Gurbel *et al.*, 1998; Opie, 2004).

The cardiomyocyte is therefore challenged on four fronts: (1.) depletion of energy necessary for survival; (2.) dysregulation of protein homeostasis; (3.) acidic intracellular conditions; and (4.) increased inward flux of fluid into the cell. These combined stressors eventually lead to cell death. Ischaemic tissue is however not homogenous. Jennings & Reimer (1983) distinguished between reversibly and irreversibly damaged cells at any one timepoint in the ischaemic heart. Also, cells follow different modes of cell death: autophagy (Matsui *et al.*, 2007; Loos *et al.*, 2011) , apoptosis (Fliss & Gattinger, 1996; Anversa *et al.*, 1998) and oncotic necrosis (Ohno *et al.*, 1998; Freude *et al.*, 2000) are potentially all three present in the ischaemic myocardium.

The clinical features of myocardial infarction were described as early as 1910 (Nabel & Braunwald, 2012). At that stage of course, treatment options were limited to bed rest and general heart ailment cures, such as digitalis (Herrick, 1912). As time progressed physicians became increasingly aware that the treatment of these patients could be optimized by a combination of effective patient monitoring (in the acute phase following the onset of symptoms) and the application of resuscitation techniques, which eventually led to the introduction of coronary care units in the 1960s (Julian, 1987). Treatment was however still largely symptomatic and primarily aimed at combating life-threatening rhythmic disturbances following infarction (Julian, 1987). At this time there was however also a growing interest in different pharmacological and mechanical interventions which could be used to improve the prognosis of MI. An in-depth discussion of these interventions falls outside the scope of this thesis and is briefly summarized in table 1.1. For an excellent review concerning the state of treatment and scientific pursuit in 1981, before the mainstream acceptance of reperfusion strategies and cardioprotection as we know it today, see Rude *et al.* (1981).

Table 1.1. Summary of the treatment regimens which were either in use, or under investigation, for the treatment of myocardial infarction before the mainstream application of reperfusion strategies.

Clinical aim	Specific mechanism	Treatment tested	Reference
To increase oxygen availability to the ischaemic zone.	• To increase blood flow through collateral blood vessels. • To reduce coronary spasm. • To limit further thrombus formation. • To increase oxygen content of the blood.	• Nitroglycerin and other nitrate preparations. • Hyaluronidase. • Ca^{2+} channel blockers. • Anticoagulants, eg. heparin. • Antiplatelets, eg. aspirin. • Steroidal anti-inflammatory drugs. • Oxygen inhalation.	• Rude *et al.*, 1981. • Kloner & Braunwald, 1987. • Ganz, 1983. • ISIS-2 group, 1988.
To reduce the oxygen demand of the ischaemic myocardium.	• To reduce contractile activity of the heart muscle. • To reduce the afterload on the heart.	• β adrenergic blockade. • Vasodilators. • Ca^{2+} channel blockers.	• Rude *et al.*, 1981. • Kloner & Braunwald, 1987. • Maroko *et al.*, 1971. • Herlitz *et al.*, 1983.
To optimally modify the metabolism of the heart.	To enhance anaerobic metabolism, while reducing the amount of free fatty acids in the circulation.	Glucose-insulin-potassium administration.	• Rude *et al.*, 1981.
To oppose the cellular derangements associated with prolonged ischaemia.	• To restore intracellular K^+ levels. • To minimize Ca^{2+} entry into the cardiomyocyte.	• Glucose-insulin-potassium administration. • Ca^{2+} channel blockers.	• Brachfeld, 1973. • Kloner & Braunwald, 1987.
To reduce inflammation.	To reduce the development of general inflammation due to the ischaemic injury.	Steroidal and non-steroidal anti-inflammatory drugs.	• Rude *et al.*, 1981.

It quickly became evident that one of the most important aspects of treating MI, was to keep the time duration between the onset of symptoms and the onset of treatment as short as possible. This was confirmed by basic science studies which revealed that as time progresses, ischaemic injury to the myocardium spreads like a wave from the subendocardium to the subepicardial myocardium (Reimer *et al.*, 1977), i.e. from deeper to more superficial layers of the heart muscle. It therefore follows that the duration of ischaemia is one of the most important determinants of the eventual extent of ischaemic damage (Reimer *et al.*, 1977; Rude *et al.*, 1981; Jennings *et al.*, 1983; Edoute *et al.*, 1983). This realization set the scene for the modern approach to myocardial ischaemia: rapid reperfusion.

Two different strategies can be followed, depending on the availability of resources, to reperfuse the ischaemic heart:

1.) Percutaneous coronary intervention (PCI): In its most basic form this technique entails the catherization of the occluded coronary artery with a catheter equipped with an inflatable balloon, which is then used to open the occluded artery. Since its first description in 1979 (Grüntzig *et al.*), it has developed to an intervention allowing the placement of a stent in the artery as well as delivering drugs directly to the myocardium (Stefanini & Holmes, 2013). Where the availability of resources allows it, it has become the intervention of choice (Keeley *et al.*, 2003), although the optimal treatment of acute MI is still a point of debate (Bogaty & Brophy, 2006).
2.) Thrombolytic treatment: This approach predates PCI and simply entails the pharmacologically mediated fibrinolysis of the obstructing thrombus (De Bono, 1987; Nabel & Braunwald, 2012). It is ideal in resource-limited settings, although it is considered less effective than PCI (Ting *et al.*, 2006).

Irrespective of the reperfusion strategy followed, the most important variable that should be kept in mind is keeping the ischaemic time as short as possible (Bogaty & Brophy, 2006; Ting *et al.*, 2006; Boden *et al.*, 2007). According to the guidelines of the American College of Cardiology / American Heart Association, where PCI is possible, reperfusion must be attained within 90 minutes from the time that the patient first made medical contact (Ting *et al.*, 2006; Boden *et al.*, 2007).

Although reperfusion is therefore considered as the best treatment for the ischaemic heart, its advent has revealed yet another challenge: reperfusion injury.

Reperfusion injury

Reperfusion injury refers to injury associated with reperfusion *per se*. The underlying causative mechanism is situated in the events associated with reperfusion and not the preceding sustained ischaemia (Piper *et al.*, 1998). This implies that at the end of an ischaemia / reperfusion (I/R) episode a portion (possibly as much as 30-50%) of the final infarcted zone was viable immediately prior to reperfusion and died during reperfusion, due to reperfusion (Yellon & Hausenloy, 2007). This is a challenging concept since “reperfusion” as a phenomenon only exists because there was a prior ischaemic episode, and it is difficult to distinguish between damage due to ischaemia and damage due to reperfusion. It is therefore not surprising that for a long time the very existence of reperfusion injury was a matter of hot debate (Przyklenk, 1997), although the relatively recent discovery of interventions applied at the onset of reperfusion and capable of limiting tissue damage has illustrated the contribution of reperfusion injury itself to the pathology associated with ischaemia and reperfusion (Piper *et al.*, 1998). Regardless of the precise definitions, it has become

undeniable that reperfusion is associated with a number of detrimental events within the cardiomyocyte:

Calcium overload

Calcium ion (Ca^{2+}) dysregulation at the onset of reperfusion could lead to contracture, compromised function and eventual cell death. Several different mechanisms have been implicated in the increased influx of Ca^{2+} into the cardiomyocyte. On the one hand the reduction in ATP availability during ischaemia leads to a reduction in the activity of the Na^+/K^+-ATPase pumps in the sarcolemma, whilst the accumulation of intracellular H^+ leads to a reversal in the direction of ion fluxes controlled by the sarcolemmal Na^+/H^+-exchanger. The net effect is an intracellular Na^+ accumulation, which in turn drives the influx of Ca^{2+} through the Na^+/Ca^{2+}-exchanger (Gumina *et al.*, 1999; Piper & García-Dorado, 1999; Inserte *et al*., 2002). More direct routes of Ca^{2+} influx have also been identified in the forms of the sarcolemmal L-type Ca^{2+}-channel (Przyklenk *et al.*, 1989) and the Ca^{2+}-ATPase located in the membrane of the sarcoplasmic reticulum (SR). In the absence of adequate ATP levels Ca^{2+}-ATPase cannot mediate the re-uptake of Ca^{2+} into the SR, while during reperfusion and re-energization the capacity of the SR may be too small to effectively remove Ca^{2+} from the cytosol (Piper & García-Dorado, 1999). It can also transpire that Ca^{2+}-ATPase becomes damaged and dysfunctional due to ischaemia (Smart *et al.*, 1997).

Irrespective of the routes followed, this catastrophic increase in intracellular Ca^{2+} levels may lead to hypercontracture (both during ischaemia and reperfusion) and is also at least partially responsible for the phenomenon of stunning (Piper & García-Dorado, 1999; Gross *et al.,* 1999). Stunning refers to the transient reduction in the functional ability of cardiomyocytes following I/R, even though the involved cells are still viable and will eventually recover (Braunwald & Kloner, 1982; Bolli, 1990).

Free radical generation

Free radicals are highly reactive chemical species with an unpaired electron in their outer orbital (Park & Lucchesi, 1999). At moderate, physiological intracellular levels they have been implicated in regulating signalling proteins (Corcoran & Cotter, 2013). However, in large quantities they become toxic to cells in that they react with, and in the process damage, all types of macromolecules: proteins, nucleic acids, membrane lipids, etc. Such an increase in free radicals, specifically reactive oxygen species (ROS), has been observed during reperfusion and contributes to the eventual injury associated with reperfusion (Ferrari *et al.*, 1990; Gross *et al.*, 1999; Zweier & Hassan Talukder, 2006).

There are several potential sources of ROS in the heart when reperfused (Park & Lucchesi, 1999), two of which are the xanthine oxidase system (Thompson-Gorman & Zweier, 1990) and the mitochondria. Mitochondria produce ROS if the supply of oxygen and electrons to the electron

transport chain is unsynchronized causing oxygen to be incompletely reduced (Zweier & Hassan Talukder, 2006).

Despite these observations that reperfusion elicits ROS generation, and the very probable detrimental effects of ROS on cardiomyocytes, their contribution to reperfusion injury is questionable in the light of several studies which have failed to show that ROS lowering interventions confer cardioprotection (Richard *et al.*, 1988; Piper & García-Dorado, 1999; Park & Lucchesi, 1999).

The mitochondrial permeability transition pore (mPTP)

Mitochondria are of critical importance in energy metabolism, free radical generation, apoptosis regulation and intracellular Ca^{2+} homeostasis. It is therefore not surprising that the patency of the mitochondria has emerged as one of the most important, if not the central, mediator of reperfusion injury.

More specifically, an increase in the permeability of the inner mitochondrial membrane (termed mitochondrial permeability transition) for any molecule smaller than 1.5 kDa has been implicated in the genesis of reperfusion-linked cell death (Halestrap *et al.*, 2007). It is accepted that this transition occurs through the opening of a specific pore (Crompton & Costi, 1988) called the mitochondrial permeability transition pore (mPTP). The exact molecular composition of the mPTP is still a matter of investigation, although cyclophilin-D (CypD) is in all likelihood somehow involved (Baines *et al.,* 2005; Nakagawa *et al.*, 2005). Recently it has been proposed that the mPTP might form between two dimerized ATP synthase molecules due to changes in the interactions between these two molecules (Bernardi, 2013). Despite this uncertainty regarding the molecular structure of the mPTP, it can be pharmacologically modulated (Halestrap *et al*., 2007; Bernardi, 2013).

Opening of the mPTP is precipitated by Ca^{2+} accumulation in the mitochondrial matrix, especially when this occurs in conjunction with an increase in the matrix concentrations of ROS and inorganic phosphates (Pi) (Crompton & Costi, 1988; Compton, 2000). Since these stimuli are also characteristic of the cellular milieu during reperfusion, it is not surprising that reperfusion, and not ischaemia, is associated with an increased tendency for the mPTP to open (Halestrap *et al.,* 2007). Opening of the mPTP is detrimental for several reasons.

Firstly the free movement of H^+ through the mPTP obviously reduces the H^+ concentration gradient across the inner membrane which is supposed to drive the synthesis of new ATP through oxidative phosphorylation. The consequence of this is not only a loss of ATP generation capability, but also an increase in the degradation of ATP as the ATP synthase in the inner membrane switches to an ATPase. Secondly, the localised depolarization accompanying the opening of a single pore will

induce opening of the surrounding mPTPs. Thus, opening of a single pore will lead to the opening of all the pores in a mitochondrion. Thirdly, while the small solutes exit the mitochondrial matrix and equilibrate with the extra-mitochondrial space, the larger osmotically active proteins remain trapped in the matrix. This leads to the development of an osmotic gradient driving the movement of water into the matrix causing it to swell and the outer mitochondrial membrane to eventually burst. This allows the release of pro-apoptotic mediators (such as cytochrome c) into the cytoplasm, thereby initiating apoptosis (Halestrap *et al.,* 2007).

The importance of the mPTP in reperfusion injury has been illustrated by the observation that maintenance of the mPTP in a closed conformation limits the development of reperfusion-linked myocardial damage (Hausenloy *et al.,* 2003*;* Kim *et al.*, 2006). Similarly the efficacy of several other cardioprotective interventions are also dependent on keeping the pore closed (Gateau-Roesch *et al.* 2006; Argaud *et* al., 2005; Bopassa *et al.,* 2006; Lim *et al.*, 2007).

Other factors contributing to reperfusion injury

As mentioned previously, ischaemia induces an increase in glycolytic flux with an associated accumulation of anaerobic metabolites, such as H^+ and lactate. This leads to a reduction in intracellular pH (acidosis). On reperfusion excess H^+ are transported out of the cell via Na^+/H^+ transporters. This is however detrimental in three ways: (1.) As mentioned before, the resulting increase in intracellular Na^+ can drive Ca^{2+} influx into the cell, contributing to Ca^{2+} overload through the Na^+/Ca^{2+} exchanger; (2.) The increase in intracellular Na^+ concentrations can contribute to the generation of an osmotic gradient across the sarcolemma favouring the inward flux of water into the cell (Inserte *et al.*, 1997; Piper & García-Dorado, 1999); and (3.) The rapid normalisation of pH constitutes the removal of a cardioprotective agent. Transient acidosis is protective since it inhibits the contractile machinery of the cardiomyocyte (thereby inhibiting the development of reperfusion contracture – Piper & García-Dorado, 1999) and keeps the mPTP in a closed conformation (if the pH of the mitochondrial matrix is also reduced - Petronilli *et al*., 1993). In this regard, transient reperfusion acidosis has been associated with cardioprotection (Kitakaze *et al*., 1988; Hori *et al.*, 1991; Fujita *et al.,* 2007; Cohen *et al.*, 2007).

Sodium ions are however not the only osmotically active species in the cells during reperfusion. As the interstitial space is more rapidly flushed of all accumulated ischaemia-related metabolites than the intracellular space, an osmotic gradient develops which leads to cell swelling and eventual damage being incurred by the sarcolemma (Tranum-Jensen *et al.*, 1981; Ruiz-Meana *et al.*, 1995; Piper & García-Dorado, 1999).

As mentioned previously, ischaemia also influences protein homeostasis by reducing the activity of the UPS, with a concomitant increase in autophagy. Reperfusion has been reported to induce a

partial recovery in proteasomal activity (Dosenko *et al.*, 2006; Hochrainer *et al.*, 2012), which might not be beneficial, since Dosenko and colleagues (2006) reported that the administration of low concentrations of proteasomal inhibitors immediately before the reoxygenation of anoxic neonatal cardiomyocytes led to a reduction in the occurrence of apoptosis and necrosis in these cells. The increase in ATP levels, as well as the degree of oxidative protein damage and ER stress associated with reperfusion are all factors which stimulate an increase in autophagy during reperfusion (Hamacher-Brady *et al.*, 2006; Matsui *et al*., 2008; Nishida *et al.*, 2009). The implications of this increase in autophagy is uncertain, with researchers arguing for both its beneficial (Hamacher-Brady *et al.*, 2006; Giricz *et al.*, 2012) as well as deleterious effects (Matsui *et* al., 2007; Matsui *et* al., 2008; Nishida *et al.*, 2009).

On a more systemic level, the inflammatory response to an ischaemic episode, and its associated tissue injury, also contribute to cardiomyocyte damage – especially since it is only during reperfusion that the inflammatory effectors can actually reach the relevant tissue. Some of the inflammatory machinery which has been implicated in I/R are the complement system (Hill & Ward, 1971; Yasojima *et al.*, 1998; Buerke *et al.*, 2006) and neutrophils (Romson *et al.*, 1983; Kin *et al,* 2006). For reviews on this see Parks & Lucchesi (1999) and Jordan *et al.* (1999).

Conclusion

Ischaemia is not simply a lack of oxygen, but also constitutes a series of perturbations due to the lack of nutrition and the accumulation of waste products, putting affected cardiomyocytes under severe stress. This stress can only be alleviated by reperfusion, which makes it the only viable treatment. Yet, at the same time, reperfusion itself initiates molecular events which eventually lead to the demise of cells already under stress, but not yet dead, due to preceding ischaemia.

The aim of this literature overview is to give the reader an overall perspective of the events associated with both ischaemia and reperfusion; it is not aimed at contributing to the debate concerning the existence and / or definition of reperfusion injury. It is however clear that reperfusion is in itself associated with a molecular identity quite different from that of ischaemia, although the two are inexorably linked. For the purposes of this thesis, I will therefore approach ischaemia and reperfusion as two distinct conditions in the cardiomyocyte, while at the same time attempting to recognise both of them as different components of a single stressor namely ischaemia / reperfusion injury.

Cardioprotection following ischaemia / reperfusion

At the moment the best treatment intervention for a patient suffering MI is rapid reperfusion. There are however limits as to how quickly this can be achieved (see Boden *et al.* (2007) for a discussion

concerning the practicalities of timely reperfusion). At the same time, we now know that reperfusion itself is associated with cell death.

These two observations lead to two questions: Firstly, can more be done additional to rapid reperfusion to treat the heart exposed to I/R? Secondly, what can be done to alleviate the detrimental effects of reperfusion, thereby enhancing the advantages of early reperfusion? These two questions lead us into the realm of cardioprotective interventions.

Cardioprotective interventions

In 1986 Murry and colleagues made the surprise observation that the administration of multiple (2 or 4) short episodes of ischaemia (10 minutes), separated by 20 minutes reperfusion, did not lead to the expected accumulation of ischaemic effects (measured as loss of adenine nucleotides and cell death) in comparison to a single 10 minute ischaemic exposure (Reimer *et al*., 1986). These intriguing results lead them to test the hypothesis that the administration of short ischaemic cycles prior to sustained ischaemia might actually protect the heart against the ensuing ischaemic stress. A hypothesis which was proven correct: 4 cycles of 5 minutes ischaemia/reperfusion reduced infarct size by 25% after 40 minutes of coronary occlusion in dogs (Murry *et al.,* 1986). Since this technique utilized brief periods of ischaemia to “prime” the myocardium to become more resistant against the detrimental consequences of a sustained ischaemic episode in the future, it was called “ischaemic preconditioning” (IPC). At that stage the discovery of IPC served to invigorate research on myocardial ischaemia because it revealed that the heart has the innate potential to protect itself against ischaemic damage. Indeed further research revealed that IPC could reduce infarct size (IFS) (Murry *et al.,* 1986) and reperfusion arrhythmias (Shiki & Hearse, 1987), as well as improve function following I/R (Cave & Hearse, 1992). Ischaemic preconditioning is still regarded by many as probably the most robust, reproducible and strongest cardioprotective intervention known.

Later it was found that the protection afforded by an IPC stimulus was biphasic: in the short term (within minutes to 2 to 3 hours) a potent cardioprotective effect was elicited which then waned away but was later (12 – 24 hours later) replaced by a less potent but longer lasting (in the order of 3 to 4 days) cardioprotective effect. This second stage of protection is called delayed, or late preconditioning, or simply the second window of protection (SWOP) and can be ascribed to protein expression dependent mechanisms which are recruited by the initial short periods of ischaemia (Kuzuya *et al.*, 1993; Marber *et al.*, 1993; Bolli, 2000; Yellon & Downey, 2003).

Interestingly Przyklenk and colleagues (1993) found that the application of brief cycles of ischaemia to the vascular bed supplied by the circumflex branch induced protection to an ischaemic episode in the tissue supplied by the descending coronary artery. Protection was therefore being “communicated” between vascular beds in the heart. This concept was later

extended to show that IPC of other organs or limbs can also confer resistance to the heart against an ensuing sustained ischaemic episode (Gho *et al.*, 1996). This form of intervention is called remote ischaemic preconditioning (RIPC), and it reveals that at least a degree of the protection elicited by brief ischaemic episodes must be mediated by some form of systemic communication system – the nervous system and / or a blood borne chemical messenger (for a review concerning RIPC see Tapuria *et al.* (2008)).

In 2003 the group of Vinten-Johansen (Zhao *et al.*, 2003) described a phenomenon similar to IPC, but with the critical difference that short cycles of I/R were administered at the very onset of reperfusion. Postconditioning (PostC), as it became known, utilized shorter and more numerous ischaemic cycles than IPC and also proved to be less potent and consistent in the protection that it evoked (Tang *et al.*, 2006; Dow & Kloner, 2007). It however has the clinical edge on IPC, since it is a cardioprotective intervention which can be applied after sustained ischaemia and therefore falls within the exact timeframe when a patient would present for treatment following an MI. PostC also highlighted reperfusion as a period which could be (and should be) targeted for cardioprotection (Vinten-Johansen *et al*., 2005; Downey & Cohen, 2005). The fact that an intervention applied at reperfusion could elicit cardioprotection also confirmed the existence of reperfusion injury *per se* (Piper *et al.*, 1998). Interestingly most researchers found that the very first moments of reperfusion is the critical timepoint to intervene to protect the heart (Kin *et al.*, 2004; Yang *et al.*, 2004), although it has been reported that interventions at a later stage could also still limit I/R injury (Roubille *et al.*, 2011).

These cardioprotective manoeuvres have been thoroughly investigated in the basic science setting and have even been shown to benefit the human heart exposed to I/R. Ischaemic preconditioning is in its nature limited in its potential application to the MI scenario, since it needs to be applied prior to ischaemia. It has however been applied with success in more controlled situations, such as coronary artery bypass grafting (CABG), where the onset of ischaemia can be controlled (Yellon *et al*., 1993; Teoh *et al.*, 2002). As an intervention applied during the first moments of reperfusion, postconditoning has been identified as potentially clinically relevant. Already in 2005 it was proven to be effective in the human heart (Staat *et al.*, 2005). Other studies soon followed which also confirmed its potential benefits in the human heart (Ma *et al.*, 2006; Yang *et al.*, 2007). The clinical applications of remote ischaemic preconditioning has also been explored, especially since RIPC allows for the non-invasive application of a preconditioning stimulus (in the form of limb ischaemia/reperfusion applied by a pressure cuff) to protect the heart (Schmidt *et al.*, 2012). Emboldened by the successes of PostC, researchers have bypassed the limitation of having to apply the conditioning stimulus before ischaemia, by rather applying the conditioning stimulus during sustained ischaemia (Bøtker *et al.*, 2010).

Mechanical intervention in already diseased coronary arteries, as required by "ischaemic conditioning", is however a risky endeavour. However, these different "ischaemic conditioning" interventions exposed the heart's own innate capacity to become more resistant to the detrimental effects of I/R – a capacity which could be exploited by more acceptable pharmacological means to induce cardioprotection. The question which however first needs to be answered is what is the mechanism(s) whereby "ischaemic conditioning" elicits cardioprotection?

Mechanisms of cardioprotection

A considerable body of research has already been done in the pursuit of this question and is reviewed in detail by Yellon & Downey (2003); Hausenloy & Yellon (2007); and Zhao & Vinten-Johansen (2006). For the purposes of this introduction a very brief overview of what is currently known will be given, with the focus on specifically IPC and PostC.

Reducing inflammation

The original descriptions of both IPC and PostC were done in *in vivo* experimental models which revealed that these cardioprotective interventions elicit some of their protective effects by reducing the systemic inflammatory response to I/R. In this regard it has been reported that IPC reduces neutrophil accumulation during reperfusion (Bufkin *et al.*, 1998; Wang *et al.*, 1999). PostC has also been shown to reduce neutrophil accumulation in the area at risk, as well as tissue oedema in the ischaemic epicardium (Zhao *et al.*, 2003; Kin *et al.*, 2004; Mykytenko *et al.*, 2007). Recently Xiong and colleagues reported (2011) that both IPC and PostC reduced serum levels of tumour necrosis factor-α (TNF-α) in an *in vivo* rat model, although the effect was more pronounced following IPC. In fact, PostC has even been found to reduce the release of TNF-α in a neonatal cardiomyocyte model (Sun *et al.*, 2006).

The observation that both IPC and PostC can be applied in the isolated heart perfused with a crystalloid buffer however implied that there are even more intrinsic mechanisms at work.

Reactive oxygen species

Both IPC and PostC have been shown to be associated with a reduction in the generation of free radicals during reperfusion (Halkos *et al.*, 2004; Zhao *et al.*, 2003; Mykytenko *et al.*, 2007; Quarrie *et al.*, 2012). This makes sense since, as discussed before, ROS production has been implicated as a contributing factor to reperfusion injury. However, the association between ROS and cardioprotection is not that simple. Oxygen radicals have also been implicated as important components involved in triggering IPC-mediated protection (Baines *et al.*, 1997; Vanden Hoek *et al.*, 1998). Likewise in PostC, the administration of ROS scavengers prior to (Tsutsumi *et al.*, 2007) or during (Penna *et al.*, 2006) a PostC intervention abolished the protection elicited by PostC.

These findings emphasise the importance of timing and possibly also of quantity in determining the effects of ROS in the setting of cardioprotection.

Protein homeostasis

In light of the perturbations in protein homeostasis *via* the UPS and autophagy associated with I/R, it is not surprising that both these processes have been implicated in cardioprotection as well. Concerning proteasomal degradation there is both evidence for its inhibition and its activation to induce protection against I/R injury, with both the timing of modulation of UPS activity as well as the specific protein substrates involved determining the eventual outcome (Yu & Kem, 2010; Calise & Powell, 2013). It is however noteworthy that IPC activates proteasomal activity during I/R, while proteasomal inhibition abrogates the protective effects elicited by IPC and PostC (Dosenko *et al.*, 2006; Churchill *et al.*, 2010; Divald *et al.*, 2010).

Similar to the UPS, there is also still uncertainty regarding the role of autophagy in protection. Some have found that its inhibition confers protection (Valentim *et al.*, 2006), while others have reported that its activation induces an increased resistance against I/R injury (Yitzhaki *et al.*, 2009; Loos *et al.*, 2011; Sciaretta *et al.*, 2012). There are some compelling explanations for the proposed beneficial effects of autophagy, including an increase in the generation of ATP, a reduction in the number of damaged mitochondria in the cell which might contribute to the initiation of cell death, a reduction in ER stress which in turn will limit the development of apoptosis and a reduction in the acidification of the cytosol due to an increase in the inward movement of H^+-ions into the lysosomes involved with autophagy (Gottlieb RA *et al.*, 2009; Dong *et al.*, 2010; Loos *et al.*, 2011; Giricz *et al.*, 2012).

Intracellular signalling pathways

Intracellular signalling constitutes the communication pathways linking external stimuli and conditions to intracellular responses and adaptation. In the case of IPC, the cycle(s) of brief ischaemia prior to sustained ischaemia serve as the “trigger” which initiates signalling cascades which will communicate a message of adaptation to the cellular end-effectors; i.e. the cellular components which must facilitate the final cardioprotection. The way in which the “trigger” IPC stimulus “informs” the cardiomyocytes is by the release of autocrine and paracrine messengers, the most prominent of which are adenosine, bradykinin, the opioids and catecholamines. These molecules are released by the heart due to ischaemia and then bind to their receptors (which are G_i protein-coupled receptors (Gi-PCR)) in the myocardial sarcolemma.

Activation of these receptors initiate several signalling pathways. How these signalling pathways interact is not completely known, but the following model (proposed by Yang and colleagues, 2010) seems to emerge:

Binding of adenosine, bradykinin and the opioids to their respective receptors induces the activation of Protein kinase C (PKC) (Goto *et al.*, 1995; Miki *et al.*, 1998). Protein kinase C seems to be of critical importance, since PKC inhibition abolishes IPC protection (Ytrehus *et al*., 1994). Through some unknown mechanism PKC then increases the affinity of the $A2_b$ adenosine receptor ($A2_bAR$) for adenosine (Kuno *et al.,* 2007). This then enhances the signalling associated with $A2_bAR$, including the extracellular signal-regulated kinase p42/p44 (ERK p42/p44) and the phosphatidylinositol 3-kinase (PI3-kinase) – protein kinase B (PKB)/Akt pathways (Solenkova *et al.,* 2006). The involvement of these pathways has been shown both during the triggering phase (prior to sustained ischaemia) (Tong *et al.*, 2000; Uchiyama *et al.*, 2004), as well as during reperfusion (Hausenloy *et al.*, 2005) – in the context of which they are collectively known as the Reperfusion injury salvage kinase (RISK) pathway. It should also be mentioned that several different stimulants can activate the RISK pathway, not only the $A2_bARs$.

Active PKB/Akt stimulates the production of nitric oxide (NO) via the phosphorylation of endothelial nitric oxide synthase (eNOS). In turn NO can stimulate guanylyl cyclase (GC) to increase the levels of cyclic guanosine monophosphate (cGMP) which then activates protein kinase G (PKG). PKG opens the mitochondrial ATP dependent potassium channel (mK_{ATP} channel). Opening of the mK_{ATP} channel induces a burst of ROS production by the mitochondria (Cohen *et al*., 2001; Oldenburg *et al.*, 2004) which then contributes to signalling, probably by activating PKC (Korichneva *et al.*, 2002; Liu *et al.,* 2008). This would then potentially lead to a positive feedback strengthening of these protective signalling cascades.

Although the above described theory makes sense and is strengthened by experimental evidence, reality is not as simple: there are several signalling molecules not accounted for by this model, amongst them the signal transducer and activator of transcription 3 (STAT3) which has been shown to represent an alternative cardioprotective pathway to RISK (Lecour *et al.*, 2005) known as the SAFE (Survivor Activating Factor Enhancement) pathway (Lecour, 2009).

In addition, there is also the question as to the final target onto which all these signalling pathways signal. From the data it is the mitochondria, and specifically the mPTP, which emerges as the probable ultimate end-effector. As mentioned previously, opening of the mPTP during early reperfusion is detrimental. It has however been found that IPC maintains the pore in a closed conformation (Javadov *et al.*, 2003; Hausenloy & Yellon, 2007), thereby favouring survival. Juhaszova and colleagues (2004) suggested that it is possibly glycogen synthase kinase 3-β (GSK3-β) which acts as a central nexus point between several cardioprotective pathways and in some, as yet unknown way, interacts with the mitochondria to stabilize the mPTP in a closed conformation.

The mechanism behind the cardioprotection elicited by PostC utilizes exactly the same role-players, only in a new setting. Possibly the most complete model explaining PostC protection at the moment is described by Cohen *et al.*, 2007. Just as is the case with the triggering stimulus of IPC, sustained ischaemia leads to the release of opioids, adenosine and bradykinin. These molecules bind to and activate their respective receptors but, in the absence of adequate levels of oxygen, cannot stimulate the production of the ROS which is required to activate PKC. In normal reperfusion, the rapid rate of pH restoration proves detrimental in that the inhibitory effects of acidosis on the mPTP is relieved before the mPTP can be kept closed by cardioprotective signalling pathways. PostC however is simply a technique to decelerate the rate of pH normalization, while still allowing the supply of enough oxygen for ROS-dependent signalling to become suitably activated to recruit the RISK pathway to keep the mPTP closed.

We therefore see that these different forms of cardioprotection, IPC and PostC, recruit common signalling pathways to confer protection. These pathways are receptor activated and recruit multiple participators, the most important of which are PKC, PKB/Akt, ERK p42/p44, GSK3-β, and finally the mPTP in the mitochondria. This is then also the strength of IPC and PostC cardioprotection: it is not dependent on a single intracellular mediator, but rather recruits several signalling pathways and mediators in order to efficiently and robustly increase resistance to I/R injury.

Cardioprotection: a clinical reality?

It is now 27 years since the cardioprotective effects of IPC was first described. Massive gains have been made in understanding the way in which the heart reacts to ischaemia and reperfusion, how I/R injury develops and which mechanisms are naturally available to confer resistance against I/R injury. Several pharmacological mimetics and interventions have been put forward, tested and proven to also confer cardioprotection (Gerczuk & Kloner, 2012). Yet, as things stand, clinical translation of these cardioprotective interventions have not met expectations at all, in fact none of the researched and proposed cardioprotective interventions has been assimilated into standard clinical practice. This has left the research field in a conundrum and caused a shift in the field of cardioprotective research away from delineating mechanisms to application of protective interventions. In recent years many authors and working groups have attempted to address the reasons why there is a lack in the clinical translation of our basic research: see Bollli *et al.* (2004); Cohen & Downey (2011); Kloner & Longacre (2011) and Sanz-Rosa and colleagues (2012).

In an attempt to determine why cardioprotection is still confined to the laboratory Bolli and colleagues (2004) distinguished between limitations and challenges on the clinical and pre-clinical levels. A complete discussion concerning all these possibilities falls outside the scope of this thesis. It is however interesting to note some of the possible reasons put forward on the pre-clinical

level. The most disturbing phenomenon characterizing the field of cardioprotection is the lack of consistency in terms of the efficacy of an intervention between different research labs, and also between different animal species tested. This could be ascribed to inappropriate animal models. It could also be symptomatic of interventions that are simply not robust enough to work at all times and in all animals. This is a reasonable assumption, since many of the interventions targeted only one or two of the pathways involved in cardioprotection, while IPC and PostC recruit numerous pathways simultaneously.

In my opinion there is also merit in the argument that we simply do not yet understand enough of the complete signalling picture in cardioprotection. Could it be that we are missing something which could increase the robustness and strength of the pharmacological mimetics of IPC and PostC which have already been identified? As Gerd Heusch puts it in his review on the topic in Lancet (2013): “Clearly we must develop an improved understanding of cardioprotective signalling, particularly in human hearts, to progress with this approach.”

Almost all of the research concerning the problem of I/R injury has focussed on the participation of several kinases in cardioprotective signalling. These enzymes phosphorylate other proteins and enzymes thereby changing the function of their respective substrates. There is however another side to this story...

Signalling through protein phosphorylation

Protein phosphorylation is an enzyme mediated process was first described in 1953 by Burnett and Kennedy. Since then it has become increasingly evident that protein phosphorylation is a dynamic process which serves to dramatically expand the functional, spatial and temporal characteristics of proteins beyond the dynamics of protein expression and degradation. It is in fact very conceivable that in the years to come phosphorylation, and other forms of protein posttranslational modification, will rival traditional genomic based regulation of cellular proteins in terms of complexity, as well as importance for the control of cellular processes (Pawson & Scott, 2005; Porter *et al.,* 2012). This is illustrated by a recent study done by Olsen and colleagues (2006). They performed a phosphoproteomic analysis of HeLa cells exposed to endothelial growth factor (EGF) in order to map the phospho-proteins recruited by this signalling pathway, over time (the first 20 minutes following exposure), in both the cytosolic and nuclear fractions. They detected 2 244 phospho-proteins with a total of 6 600 phosphorylated residues between them, leading them to conclude that most *in vivo* phospho-sites are still to be discovered. There are only three amino acid residues which have been found to be phosphorylated: serine, threonine and tyrosine (Porter *et al.*, 2012). In Olsen’s experimental setup they found that of the phosphorylated sites detected 1.8% were tyrosine residues, 11.8% were threonine residues, while 86.4% constituted serine phosphorylation.

The level of phosphorylation of a protein is determined by the balance between the phosphorylation of that protein (mediated by a kinase, or kinases), as well as the dephosphorylation of that protein (mediated by a phosphatase, or phosphatases) (Wera & Hemmings, 1995; Hunter, 1995). Traditionally the kinases have received more attention than the phosphatases. This might be due to an earlier school of thought that the phosphatases are merely house-keeping enzymes, maintaining an ubiquitous degree of dephosphorylation. An approach which could be justified when it is taken into account that the human genome codes for approximately 518 kinases (90 tyrosine kinases and 428 serine/threonine kinases) and only about 137 phosphatases (107 tyrosine phosphatases and 30 serine/threonine phosphatases) (Shi Y, 2009). It has however come to light that the phosphatases are also, like the kinases, subject to stringent intracellular regulation. In fact, the limited number of phosphatase catalytic subunits in the genome has necessitated a degree of regulation employing more diversity and complexity than is the case for most kinases. (For a general overview of these regulatory mechanisms see Shi (2009) and Gomperts *et al.* (2009).)

As mentioned earlier, intracellular signalling in its most basic form, is the communication of events outside a cell to the interior of the cell in order for the cell to respond to the extracellular environment. As already discussed, myocardial IPC is a typical example of this: transient ischaemia is communicated to the nucleus and mitochondria of a cardiomyocyte in order to elicit adaptations to increase the resistance of the cell to I/R damage. This transfer of information is mediated by several “communicator” molecules: adenosine, the adenosine receptors, PKC, PKB, GSK3-β, etc. Fundamentally intracellular signalling is communication via sequential protein phosphorylation. In reality, the signal being communicated is “coded” for in the phosphorylation status of the communicator molecules. This is not unlike other forms of basic communication such as Morse code or binary code, where the message being communicated is coded for using only two variations – for Morse code it is “sound” or “no sound”, for binary it is “1” or “0”, and for signalling it is “phosphorylated” or “unphosphorylated” (Figure 1.1). Keeping this simplified model of signalling in mind it becomes obvious why the balance between “phosphorylation” and “dephosphorylation” is important.

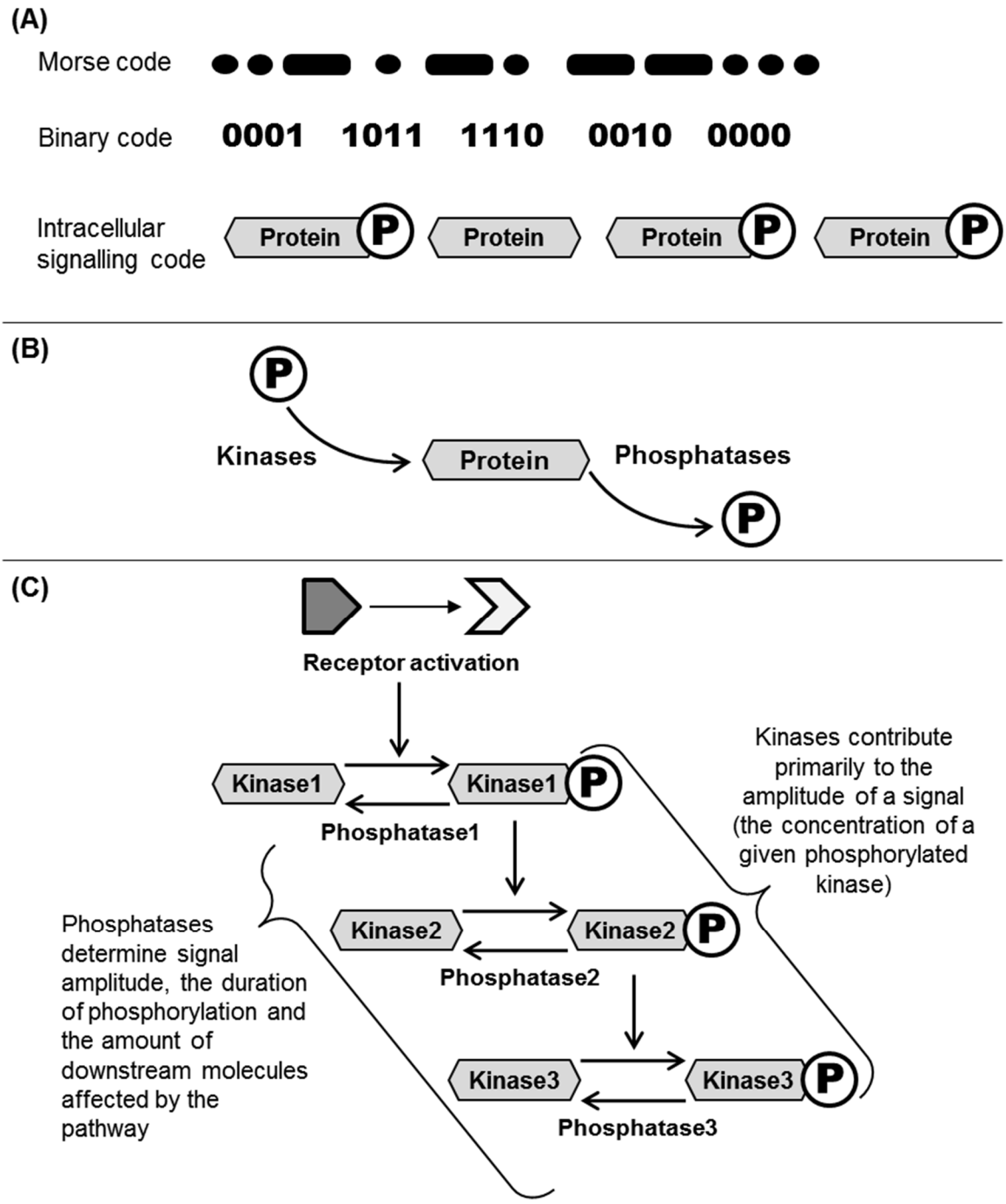


Figure 1.1. The mathematical modelling of signal transduction pathways.
(A) Signalling can be conceptualized as the communication of messages which are coded in the phosphorylation status of participating signalling machinery, akin to Morse- or binary code. (B) The level of phosphorylation of a given protein is a function of the balance between kinase and phosphatase activity directed against that protein. This implies that kinases and phosphatases are controllers of signalling. (C) Kinases primarily determine signal amplitude, while the phosphatases contribute to amplitude, signal duration and integral strength (Heinrich et al., 2002; Hornberg et al., 2005).

In the 1970's work was done on trying to describe the activity of metabolic pathways on a systems biology level using mathematical modelling, an approach called metabolic control analysis (MCA)

(Kacser *et al.*, 1995; Westerhoff, 2008). At the turn of this century this approach was applied to signalling cascades in an attempt to define the control of kinases and phosphatases involved in signal transduction (Heinrich *et al.*, 2002; Hornberg *et al.,* 2005). While this type of science falls outside the scope of this thesis, these authors came to some interesting conclusions which will be briefly mentioned.

As is the case with models such as these, they are built on certain assumptions in order to simplify the situation. For signalling cascades the following assumptions were made: (1.) the signalling pathway is a simple, linear pathway; (2.) activating processes in the pathway entail either receptor activation or kinase mediated phosphorylation of participating proteins; (3.) deactivating processes entail receptor internalization or degradation, or phosphatase mediated dephosphorylation of participating proteins; and (4.) after activation the signalling pathway will eventually return to its pre-activation state. We will focus on three parameters which were investigated:

1.) Signal duration, defined as the average period of time that a specific kinase is activated.
2.) Signal amplitude refers to the concentration of the specific kinase which is activated.
3.) Integral signal strength is a measure of the number of downstream molecules eventually affected by the pathway.

What these authors found was that their models predicted the kinases to have a more significant impact on the amplitude of a signal than the phosphatases, although phosphatase activity also contributed. Signal duration and integral strength were however primarily determined by the deactivating reactions – i.e. the phosphatases. Hornberg and colleagues (2005) went on to demonstrate these trends in an actual mitogen activated protein kinase (MAPK) / ERK kinase (MEK)-ERK signalling cascade.

These models represent an approximation of reality, but their results are thought-provoking. They indicate that the phosphatases might be more influential in determining some of the characteristics of the signal being propagated than the kinases. Even if a person is sceptical concerning these results, it must be conceded that the phosphatases might be more important in signalling than initially appreciated.

If this is in fact true, then the contribution of the phosphatases to cell survival signalling and cardioprotection must be pronounced and may therefore be a good target for the modulation of I/R injury treatment.

The protein phosphatases and myocardial ischaemia / reperfusion

Targeting protein phosphatases in cardioprotection

As mentioned previously, there are only three amino acid residues which have been found to be phosphorylated: serine, threonine and tyrosine. Reversal of phosphorylation is accomplished by three broad families of phosphatases: the protein tyrosine phosphatases (PTPs), the dual specificity phosphatases (DSPs) and the protein serine/threonine phosphatases (PSPs) (Gomperts *et al.,* 2009). According to Gomperts *et al.* (2009) the DSPs can be classified as a subfamily of the PTPs since they contain a conserved PTP signature motif in their structure. They however have the ability to dephosphorylate all three residues. One of the better known subdivisions of this family are the MAPK phosphatases (MKPs), which specifically catalyze the dephosphorylation of both tyrosine and threonine residues (Bermudez, *et al.*, 2010).

In 1994 Kobryn & Mandel reported that renal tissue anoxia was associated with a reduction in kinase activity, which is to be expected as ATP levels decrease, while protein phosphatase activity (specifically the PSPs Protein phosphatase 1 (PP1) and Protein phosphatase 2A (PP2A)) remained relatively unchanged. These findings imply that dephosphorylation processes will be favoured during ischaemia. Depending on the specific substrate involved, these changes in phosphorylation might be important in I/R injury. It is outside the scope of this thesis to report on the probable involvement of all the known protein phosphatases in I/R and cardioprotection. I will however highlight some examples of the work that has been done to illustrate the possible importance of phosphatase activity in these settings.

In view of all the research which has focussed on the importance of the activity of pro-survival kinases in cardioprotection it is perhaps not surprising that phosphatase inhibition in general seems to be associated with better outcomes following I/R. For example, in 1997 Xiuhua and colleagues found that the administration of okadaic acid, an inhibitor of PP1 and PP2A, prior to the hypoxia and reoxygenation of vascular smooth muscle cells led to an increase in cell viability, while the administration of a phosphatase activator (2,3-butanedione monoxide (BDM)), prior to an IPC protocol, alleviated the protective effects of IPC. These authors speculated that the effects observed were due to the interaction of these phosphatases with PKC, although they did not directly investigate this link. Armstrong and co-workers (1998) however refuted this speculation when they found that the cardioprotection elicited by the inhibition of PP1 and PP2A was not dependent on the activity of PKC in their model of isolated rabbit cardiomyocytes. They administered the phosphatase inhibitors (OA, Calyculin A and fostriecin) prior to simulated ischaemia, or after 75 minutes of ischaemia. Surprisingly, phosphatase inhibition was found to be still beneficial, even when initiated so late in ischaemia. Weinbrenner *et al*. (1998) made similar

observations. In their studies using the isolated rabbit heart, as well as isolated rabbit cardiomyocytes, the inhibition of PP1 and PP2A (by the administration of fostriecin) was found to be associated with a reduction in cell death, even when administered after the onset of ischaemia. Somewhat surprisingly though, they found that IPC did not exert an effect on the activity of either these phosphatases – thereby excluding PP1 and PP2A from the IPC-mediated mechanism of protection. In a separate study, Weinbrenner *et al*. (1998) reported similar results using inhibitors for the PSP protein phosphatase 2B (PP2B, also commonly known as calcineurin (CaN)). Interestingly they found that although the administration of the inhibitor Cyclosporine A (CsA) 10 minutes after the onset of 30 minutes regional ischaemia conferred an infarct sparing effect, this protective effect was lost if the administration occurred too late in ischaemia. It is important to note at this point that CsA is currently predominantly utilized in cardioprotection research as a pharmacological inhibitor of the formation of the mPTP. It could therefore be that these results are due to its effects on the mitochondria rather than PP2B. That being said, the observation that "early" administration of the drug during ischaemia was more beneficial than "late" administration, could be indicative of effects on the mediators of protection and not necessarily on the end-effector, in this case the mPTP which is expected to play a role during reperfusion only. Weinbrenner *et* al. also found that the inhibition of PP2B with FK506 administered as pre-treatment conferred cardioprotection. Confirming these early studies, Feng *et al*. (2011) reported that BK506 pre-treatment prior to *in vivo* regional ischaemia limited infarct size and apoptosis, probably through the suppression of PP2B and apoptosis signal-regulating kinase 1 (ASK1).

In both studies of Weinbrenner *et al.* phosphatase inhibition was applied for the whole duration of ischaemia. These studies therefore showed that PP1, PP2A and PP2B were possibly involved in the events associated with ischaemia and could be potential targets for intervention during ischaemia – at that stage (and still) a very exciting prospect. In 2002 Isotani and co-workers extended the applicability of phosphatase inhibition into the reperfusion phase: they found that the administration of OA within the first minutes of reperfusion protected rat kidneys exposed to 45 minutes of *in vivo* renal ischaemia followed by up to 24 hours of reperfusion.

Much of the earlier work was done on PSPs such as PP1, PP2A and PP2B; more recently the tyrosine and dual specificity phosphatases have also received attention, as will be discussed below.

Mackay and Mochly-Rosen (2000) employed vanadate as a PTP inhibitor and found that it was associated with an elevation in p38 MAPK phosphorylation in isolated rat cardiomyocytes, which when combined with ischaemia was associated with an increase in lactate dehydrogenase (LDH) release, indicating cell injury. These findings implicated tyrosine phosphatase activity as an

important determinant of p38 activity and therefore also cell injury. However, as we've already established, in I/R timing is everything…

Takada *et al.* (2004) found that the reperfusion administration of orthovanadate, a PTP inhibitor, following 30 minutes regional ischaemia exerted an infarct sparing effect at 24 hours reperfusion and improved function at 72 hours reperfusion in an *in vivo* rat model. They ascribed this protective effect to the activation of PKB/Akt and its downstream effectors such as GSK3-β and Bad. In 2008 Bhuiyan *et al.* showed that reperfusion administration of another vanadate molecule, vanadyl sulphate, following 30 minutes of regional ischaemia, reduced infarct size and the degree of apoptosis concomitant with positive effects on cardiac function in an *in vivo* rat model of I/R. They propose that these positive effects are mainly due to vanadyl sulfate mediated phosphorylation and activation of PKB/Akt. Expanding on these findings, Bhuiyan & Fukinago wrote a review in 2009 concerning the cardioprotective effects of vanadium compounds. Their group has repeatedly found that vanadium compounds exert cardioprotective effects in the setting of I/R and proposed that these protective effects are due to the inhibition of the PTPs responsible for the dephosphorylation of receptor tyrosine kinases, which eventually leads to an increase in the phosphorylation and activity of downstream PKB/Akt. Linking with this line of investigation, Sandin *et al.* (2011) reported that 30 minutes hypoxia followed by 30 minutes reoxygenation of NIH3T3 fibroblast cells was associated with an increase in the oxidation of the PTPs, in the process reducing their activity. One of the PTPs which were specifically investigated by this group was SHP-2, an upstream regulator of MAPK signalling. Administration of an antioxidant also reduced the reoxygenation associated phosphorylation of ERK p42/p44; supposedly through an increase in the activity of SHP-2. This illustrates an inherent mechanism aimed at favouring pro-survival signalling following I/R.

Comparatively little work has been done on the effects of the MKPs (a member of the DSP family) in the setting of I/R. Fan and co-workers (2009) addressed this void by testing the effects of the MKP-1 activator dexamethasone on infarct size (following *ex vivo* regional ischaemia) and functional recovery (following *ex vivo* global ischaemia) in a rat model. They found that dexamethasone administered intraperitoneally or directly to the perfusate, prior to ischaemia or during the onset of reperfusion, reduced infarct size and increased functional recovery. These beneficial effects were associated with an increased expression of MKP-1 (in the intraperitoneally treated group), as well as a reduction in the phosphorylation of p38 MAPK. These observations confirm the detrimental consequences of p38 MAPK phosphorylation during reperfusion (Omar *et al.*, 2012). This study of Fan *et al.* serves as a reminder that phosphatase activation is not always detrimental - the substrate targeted determines the effects. Interestingly the study by Omar *et al.* (2012) concerning p38 MAPK activity implicated p38 MAPK as a downstream substrate of either PP1 and/or PP2A, illustrating the complexity of kinase and phosphatase interaction.

Another PSP which has recently also received more attention is protein phosphatase 2C (PP2C). Protein phosphatase 2C, along with pyruvate dehydrogenase phosphatase, belongs to a Mn^{2+}/Mg^{2+}-dependent sub-family of PSPs (Gomperts *et al.,* 2009). In 2006 Zhang & Herman characterized some of the characteristics of the protein 'apoptosis repressor with a CARD domain' (ARC) in H9c2 cardiomyoblasts exposed to oxidative stress. They found that ARC exerted an anti-apoptotic effect which is dependent on it being phosphorylated on a threonine residue. They then went on to identify PP2C as a possible inactivator of ARC through the dephosphorylation of this site. This implicates PP2C as a pro-apoptotic regulator involved in oxidative stress.

PH domain leucine-rich repeat protein phosphatase (PHLPP), a member of the PP2C family, has also been implicated in I/R injury. Miyamoto *et al.* (2010) found that silencing PHLPP in neonatal rat ventricular myocytes led to an increased phosphorylation of PKB/Akt at specifically ser473 following stimulation by the administration of insulin-like growth factor-1 (IGF-1) or sphingosine-1-phosphate (S1P). This increased phosphorylation was linked to an increase in the activity of PKB/Akt as well as enhanced leukemia inhibitory factor (LIF) mediated cardioprotection following exposure to doxorubicin. This pattern was maintained in PHLPP knockout mice. Exposure of these animals to 30 minutes regional ischaemia and 120 minutes reperfusion was associated with a reduction in infarct size concomitant with an increase in the ser473 phosphorylation of PKB/Akt (measured at 30 minutes reperfusion). This study therefore identified PHLPP as a negative regulator of PKB/Akt.

Not all studies have however implicated phosphatase activity, or the inhibition thereof, in cardioprotection. Hausenloy *et al.* (2002) administered the PP2B inhibitor FK506 at the onset of reperfusion following 35 minutes of regional ischaemia in the isolated rat heart and found that it exerted no effect on infarct size. Similarly Fan and colleagues (2010) reported that administration of the drug cantharidin to isolated rat hearts at a concentration where both PP1 and PP2A is inhibited, exerted no cardioprotective effects, either as a pre-treatment or during reperfusion. Similarly, OA administration at a PP2A-specific concentration failed to confer any protection.

Despite these negative results, most studies implicate the participation of all three families of protein phosphatases in the events associated with myocardial ischaemia and reperfusion. As more research is done regarding the phosphatases, a larger and more complex signalling network of interconnecting kinases and phosphatases is being revealed.

Inducing cardioprotection requires targeting multiple molecules and pathways

A study of the events and mechanisms underlying myocardial I/R injury and the attempts that have been made to harness these mechanisms to pharmacologically protect the heart reveal the following:

1.) Ischaemia / reperfusion injury encompasses a multitude of intracellular mediators and end-effectors. Injury is associated with several aspects, such as mitochondrial dysfunction, Ca^{2+} dysregulation, pH perturbation, oxidative stress and metabolic adaptations. Each of these aspects involves numerous molecules including enzymes, membrane channels, chaperones, cytoskeletal proteins, contractile machinery, etc.

2.) Such a complex series of events ultimately require a complex and intricate solution. Ischaemic conditioning provides that solution by recruiting multiple signalling pathways to signal to divergent targets such as the nucleus, the mitochondria, sarcolemmal channels and carriers, etc. No wonder that attempts to mimic this type of "cellular systemic" phenomenon by manipulating single role-players in the process have proven disappointing.

In order to increase the robustness and efficacy of the cardioprotective interventions that have already been identified, we must search for yet more signalling regulators to manipulate alongside existing cardioprotective interventions in an attempt to enhance the strength and reproducibility of cardioprotection. In view of the work done by Heinrich *et al.* (2002) and Hornberg *et al.* (2005), as well as the laboratory work referred to above, the protein phosphatases might be those signalling regulators which may be additionally targeted in the search for robust, clinically-applicable cardioprotective interventions. The following studies illustrate this possibility by demonstrating that the phosphatases are involved in determining the characteristics of induced cardioprotection and can therefore be used alongside existing cardioprotective interventions to broaden the applicability of these interventions to situations where their efficacy is reduced.

Protein phosphatases can modify the characteristics of cardioprotection

'Phosphatase and TENsin homologue deleted from chromosome 10' (PTEN) is a DSP of which its lipid phosphatase activity has been best described. Essentially it is involved in dephosphorylating 3-phosphoinositide, thereby maintaining it at low levels. As such it is also involved in the dephosphorylation of phosphatidylinositol (PI)-3,4,5- triphosphate (PIP3) to PI-4,5-diphosphate (PIP2), a reaction which is also involved in the activation of PKB/Akt. The activation of PKB/Akt is mediated by PIP3, which is synthesized by PI3-kinase (Gompers *et al.*, 2009). PTEN is therefore an antagonist of PI3-kinase and associated with the inhibition of PKB/Akt. In 2005 Cai and Semenza investigated the possible role of PTEN in IPC. In their isolated rat heart model they found that during the brief reperfusion period following the IPC trigger / stimulus ischaemic exposure,

PTEN activity and content was downregulated in conjunction with an increase in the activity of PKB/Akt. This was however transient, and over time PTEN re-accumulated and antagonized PKB/Akt activation, thereby acting as a “timer”-switch triggering the decay of the acute protective effect elicited by an IPC trigger-ischaemic exposure. Inhibition of PTEN could therefore theoretically increase the short-term duration of protection elicited by a IPC stimulus.

PTEN has also been implicated in setting the threshold of protection. Siddall *et al*. (2008) found that in a mouse model of PTEN haplo-insufficiency an IPC protocol of 4 x 5 minutes cycles I/R reduced IFS, while in the littermate controls this protocol was still insufficient to induce protection – it was only at 6 x 5 minutes I/R that IPC elicited an infarct sparing effect in this group. Interestingly it seemed as if this reduction in protection threshold associated with reduced PTEN activity was due to an elevation in the phosphorylation of thr308 in PKB/Akt. This is in contrast to Miyamoto *et al.* (2010) who found that PHLPP inhibition enhanced ser473 phosphorylation in PKB/Akt, associated with an infarct-sparing effect. This implies that two different phosphatases, PTEN and PHLPP, regulate the phosphorylation of two separate amino acid residues in PKB/Akt. Regardless of this, the silencing of PHLPP enhanced the cardioprotective effect of leukemia inhibitory factor (LIF) in cardiomyocytes exposed to the cardiotoxic agent doxorubicin, by favouring the ser473 phosphorylation of PKB/Akt. Inhibition of both PTEN and PHLPP has therefore been shown to increase the strength of a co-applied cardioprotective intervention.

These three studies therefore illustrate the potential for phosphatase modulation to modify and enhance the efficacy of cardioprotective interventions.

Protein phosphatases in conjuction with other cardioprotective interventions

The ability to optimize the protection conferred by a cardioprotective intervention is especially desirable in situations where baseline conditions do not favour cardioprotection, such as old age and obesity.

Protecting the aged heart

Fenton and colleagues (2005) found that ventricular PP2A activity was elevated by approximately 48% under baseline conditions in aged compared to young rats. Following 45 minutes of regional ischaemia and 3 hours reperfusion this difference increased to 82%. Ischaemic preconditioning also failed to exert a cardioprotective effect in aged hearts. However, the inhibition of both PP1 and PP2A by the addition of OA during the IPC cycles restored the protective ability of IPC, although not to the same degree as seen in young hearts (Fenton *et al.*, 2005). Similarly, Przyklenk *et al*. (2008) reported that old mouse hearts could not be postconditioned. In their model, the aged hearts also presented with an increased expression of MKP-1. Administration of sodium

orthovanadate, a non-specific MKP-1 inhibitor, for 45 minutes prior to sustained ischaemia and for the first 10 minutes of reperfusion in conjunction with a PostC protocol reduced the expression of MKP-1 and restored the protective ERK p42/p44 signalling and infarct sparing effects associated with PostC in the aged hearts – again not to the same degree as in young hearts, but significantly so. Both these studies show that aging is associated with an increase in cardiac phosphatase activity which, at least partially, reduces the cardioprotection associated with ischaemic conditioning.

Protection in obesity

In a genetic model of obesity (the leptin deficient *ob/ob* mouse) Bouhidel *et al.* (2008) found that PostC also failed to confer cardioprotection relative to controls. This loss of protection was associated with the absence of pro-survival signalling activation by PostC, as determined by the phosphorylation of PKB/Akt, ERK p42/p44, p70S6K1 and AMP-activated protein kinase (AMPK) and an increase in the levels of the phosphatases PP2A, MKP-3 and PTEN. Interestingly, at baseline, the *ob/ob* mice presented with elevated pro-survival signalling with an associated reduction in phosphatase levels. Postconditioning itself elevated the phosphatase levels relative to *ob/ob* control which did not receive PostC. These results are difficult to explain and it could be argued that a genetic model of obesity is not ideal. In principle though, this study shows that obesity could be associated with the dysregulation of the protein phosphatases – making them possible targets for pharmacological intervention in an attempt to restore the cardioprotective characteristics of PostC in obesity.

These studies all illustrate the potential of phosphatase modulation to enhance the cardioprotection elicited by ischaemic conditioning. We do not propose that the protein phosphatases constitute new targets for cardioprotection, rather they present adjunct targets which may enhance the efficacy of current cardioprotection interventions.

Summary and contextualization: Why the phosphatases need to be studied in the setting of ischaemia/reperfusion

Ischaemic heart disease is a global health problem, and indeed the foremost single contributor to mortality – in both developed and developing countries. Myocardial ischaemia and its deleterious effects have been studied since the 1970's. Clinical treatment of acute myocardial infarction by reperfusion of the affected zone, using either pharmacological thrombolysis or percutaneous coronary intervention, was also developed in that time and is still the mainstay treatment option. In light of the advanced reperfusion techniques that have been refined over the past three decades we are left with two factors that still exert negative effects on clinical outcomes: (1.) the time delay between the onset of ischaemia and reperfusion; and (2.) reperfusion injury. These two factors now drive the development of cardioprotective interventions that could be applied alongside standard

reperfusion protocols. The discovery of ischaemic conditioning (IPC, PostC, RIPC and SWOP) exposed the natural innate cardioprotective capacity of the heart. Studies to delineate the mechanisms behind this cardioprotective capacity have revealed a common, but intricate, signalling network mediating protection. Unfortunately ischaemic conditioning, and its pharmacological mimetics, have not been translated to clinical practice. Many reasons for this have been put forward, amongst them the inconsistent cardioprotective effects which have been reported in the laboratory. This author believes that a part of the problem is ignorance regarding the complete signalling networks that are recruited by cardioprotective interventions. One of the very important components of signalling which has largely been ignored is the role of protein phosphatase activity. Recently it has been postulated that the phosphatases might contribute as much, or more, than the kinases to the characteristics of a signalling network. Studies that have already been done have confirmed the potential importance of the phosphatases.

In light of the importance of signalling in cardioprotection, and the fact that the role of the phosphatases in myocardial signalling is not as well documented as some of the kinases, we decided to focus our attention on the significance of phosphatase activity within some of the cardioprotective signalling cascades.

There are however numerous protein phosphatases in the heart, potentially targeting a vast array of substrates. For the purpose of this study it was decided to select a single phosphatase, focusing on a single class of substrates.

Selecting a phosphatase

As already mentioned, the majority of phosphorylated residues in HeLa cells following EGF stimulation are serine residues (86.4%), followed by threonine (11.85) and lastly tyrosine residues (1.8%) (Olsen *et al.*, 2006). This observation immediately casts the spotlight on serine and threonine phosphorylation, and by implication also dephosphorylation. Serine and threonine residues can be dephosphorylated by either DSPs or PSPs. It has however been reported that up to 90% of serine/threonine phosphatase activity in mammalian cells is catalysed by PP1 and PP2A only (Oliver & Shenolikar, 1998). The major contribution of these two PSPs to cellular phosphorylation becomes evident when both are inhibited. Neumann *et al.* characterized the effects of okadaic acid (1993) and cantharidin (1995), both inhibitors of PP1 and PP2A, on isolated guinea pig cardiomyocytes. They found that inhibition of PP1 and PP2A led to an increase in numerous, unidentified proteins, as visualized by a smear of phosphorylated proteins separated by electrophoresis in a 10% polyacrylamide gel. However Ingebritsen *et al.* (1983) reported that there was much more PP2A than PP1 present in the heart. Foulkes & Jefferson (1984) also measured 50% more PP2A activity than PP1 in the heart. In fact, according to Shi (2009), in some tissues up to 1% of total protein content can be attributed to PP2A.

In light of its cellular abundance and significant contribution to serine/threonine phosphatase activity we decided to focus our study on describing the participation of PP2A in myocardial I/R injury and determining its importance in this situation.

Protein phosphatase 2A: a brief introduction

For an excellent review concerning PP2A, see Janssens & Goris (2001). PP2A is a ubiquitously expressed phosphatase that has been implicated in a wide variety of processes ranging from development, cell proliferation and cell death, cytoskeletal dynamics, regulation of the cell cycle and the regulation of several signalling pathways. It is the extremely complex regulatory mechanisms of this enzyme (discussed in Chapter 3) which allows it to be involved in numerous divergent cell functions.

Cardioprotection

As already mentioned, the inhibition of PP2A has been found to confer cardioprotective effects, although in most of these studies PP1 was also inhibited alongside PP2A (Xiuhua *et al.*, 1997; Armstrong *et al.*, 1998; Weinbrenner *et al.* 1998; Isotani *et al.* 2002). Other studies not yet mentioned include Barancik and co-workers (1999) who also reported that the inhibition of both PP1 and PP2A by OA administration directly prior to sustained ischaemia conferred an infarct sparing effect in their *in* vivo pig model of regional ischaemia. An earlier study by Armstrong *et al.* (1997) reported that the pre-treatment of isolated cardiomyocytes from both pigs and rabbits with fostriecin at PP2A-specific concentrations elicited protection similar to IPC – thereby implicating specifically PP2A as a possible target for cardioprotection. In agreement with this, Sariahmetoglu *et al.* (2012) reported that OA administration at a PP2A specific concentration before and after 20 minutes of global ischaemia, in an isolated rat heart model, was associated with a significant improvement in functional recovery. Opposing these studies however, Fan *et al*. (2010) reported that inhibition of PP1 and PP2A using cantharidin, as well as inhibition of PP2A alone using OA, failed to elicit any cardioprotective effect on either IFS or functional recovery in an isolated rat heart model. In fact, they found that administration of these drugs prior to ischaemia was associated with mechanical failure during reperfusion.

These divergent results as well as the use of inhibitors which might also influence PP1, necessitate further clarification concerning the cardioprotective potential of PP2A.

PP2A in pathological conditions

Protein phosphatase 2A has also been implicated in chronic pathological conditions, such as diabetes and cardiac hypertrophy. Interestingly, initial work showed no association between either PP1 or PP2A and diabetes in the heart (Foulkes & Jefferson, 1984). However, more recently

Rastogi and co-workers (2003) found that in hearts from streptozotocin-induced diabetic rats both protein serine/threonine phosphatase activity, as well as PP1 and PP2A protein content, was elevated. Interestingly, PP1 expression was increased first (1 week after diabetic induction), followed by PP2A after 4 weeks. Similarly Katare *et al.* (2011) found that PP2A levels were increased in left ventricular tissue of streptozotocin-induced diabetic rats – although in their model these changes were only evident after 12 weeks.

An association between cardiac hypertrophy and PP2A has also been shown. In 2000 Bokník and colleagues found that in a cardiac hypertrophy model of chronic β-adrenergic stimulation the activities of both PP1 and PP2A were elevated and contributed to a reduction in the phosphorylation of phospholamban (PLB). In accordance with these results, Zhang *et al.* (2002) found that the overexpression of a nuclear isoform of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) led to hypertrophy in mice which was also characterized by a reduction in PLB phosphorylation in association with an increase in the expression and activity of PP2A. In a reverse approach, Gergs *et al*. (2004) characterized a model of the overexpression of the catalytic subunit of PP2A (PP2A-C) which they found presented with cardiac hypertrophy and dilatation in conjunction with reduced phosphorylation of PLB, the inhibitory subunit of troponin (TnI) and eukaryotic elongation factor 2. In contrast to these results, Huang *et al.* (1999) reported that in their model of post-infarction remodelled hypertrophy the activity of only PP1 was increased, which was in association with a reduction in the phosphorylation of PLB. Wjinker *et al.* (2011) reported similar results in human ischaemic cardiomyopathy tissue: PP1 activity was increased, while PP2A remained unchanged. This implies that different models of hypertrophy recruit different intracellular adaptations. In line with this, dilated cardiomyopathy seems to be associated with reduced expression of PP2A-C (Wjinker *et al.*, 2011; Walker *et al.,* 2013).

There is therefore a trend for the involvement of PP2A as a contributor to pathological conditions in the heart. This might lead one to think that chronic PP2A inhibition could be a protective intervention. PP2A is however necessary for survival and knock-out of PP2A-C proves lethal at embryonic level (Götz & Schild, 2003). Linking with this, silencing of the PP2A scaffold subunit (PP2A-A) in PC3-6 cells led to reduction in cell viability at day 4, with almost complete cell loss at day 8 (Strack *et al.*, 2004; Van Kanegan *et al.* 2005).

As is to be expected from an enzyme presenting such a major contribution to cellular protein dephosphorylation, numerous substrates for PP2A have been identified. We will briefly touch on some of the more important ones in the setting of the heart.

Calcium regulation and contraction

Studies regarding PP2A in hypertrophy identified PLB, an important regulator of Ca^{2+} movement into the sarcoplasmic reticulum, as one of the substrates of PP2A (Bokník *et al*., 2000; Zhang *et al*., 2002; Gergs *et al*., 2004). Protein phosphatase 2A has however also been identified as part of the macromolecular complex regulating the ryanodine receptor-2 (RyR2). This complex also contains PP1 and protein kinase A (PKA) (Marx *et al.*, 2001). Interestingly, Terentyev and colleagues (2003) found that both PP1 and PP2A could induce an increase in spark-mediated Ca^{2+} release from the SR, thereby depleting its Ca^{2+} stores. Regulation of the sodium/calcium exchanger has also been linked to a macromolecular protein complex containing PP2A, as well as PP1, PKA and PKC (Schulze *et al*., 2003). The research of Dubell *et al.* initially (2002) showed that both PP1 and PP2A contributed to steady-state L-type Ca^{2+} channel activity, although in later work (2004) they specifically identified PP1 as the relevant phosphatase. However, Shi *et al.* (2012) recently showed that PP2A physically interacts with the L-type Ca^{2+} channel, $Ca_v1.2$, and that inhibition of PP2A is associated with increased flux through this channel thereby illustrating that PP2A contributes to the regulation of this channel.

Ke and colleagues (2010) reported that p21-activated kinase-1 (Pak-1) is an upstream regulator of PP2A which under bradykinin stimulation activates PP2A, leading to the dephosphorylation of PLB as well as cardiac troponin I (TnI). Dephosphorylation of the latter leads to an increase in the Ca^{2+} sensitivity of the myofilaments. The interaction between PP2A and TnI has also been shown by others (Gergs *et al.*, 2004; Deshmuk *et al.*, 2007). It is interesting to note that this study by Ke *et al.* implicated the recruitment of PP2A into a signalling pathway which is associated with cardioprotection (i.e. bradykinin receptor stimulation).

Taken together these studies illustrate the importance of PP2A in the regulation of Ca^{2+} homeostasis and contraction.

Apoptosis

For a review on the participation of PP2A in apoptosis-related signalling see Van Hoof & Goris (2003). Most of the work done on PP2A in this setting has been done in other tissues than the myocardium, although the same, or at least similar, apoptotic signalling should occur in the stressed myocardium. In general, PP2A has been identified as a mediator of apoptosis by dephosphorylating and thereby activating several pro-apoptotic mediators such as Bad (Chiang *et al.*, 2001 & 2003; Ray *et al.*, 2005) and Bax (Xin *et al.*, 2006); as well as dephosphorylating, and in the process inhibiting, anti-apoptotic mediators, specifically Bcl-2 (Deng *et al.,* 1998; Ruvolo *et al.*, 1999; Tamura *et al.*, 2004; Ray *et al.*, 2005). PP2A has also been implicated as a pro-apoptotic regulator of other signalling molecules involved in the regulation of apoptosis. These include the transcription factor FOXO (Forkhead box, class O) which is dephosphorylated by PP2A, allowing it

to stimulate the upregulation of pro-apoptotic Bim (Yan *et al.*, 2008). Several studies have also found that PP2A induces apoptosis by dephosphorylating and inactivating the pro-survival ERK p42/p44. Intriguingly, p38 MAPK has been suggested as an upstream activator of PP2A in this pro-apoptotic signalling cascade (Westermarck *et al.*, 2001; Li *et al.*, 2003; Grethe & Pörn-Ares, 2006) which has also been identified in cardiac myocytes (Liu & Hofmann, 2004). Although these studies all implicated PP2A as a pro-apoptotic mediator, Boudrea *et al.* (2007) reported that PP2A inhibition in Jurkat- and T leukemia cells promoted apoptosis within 18 hours after the onset of inhibition, via a mechanism that involved the permeabilization of the mitochondrial membrane. Protein phosphatase 2A therefore emerges as an important role-player in the regulation of apoptosis, both as a pro-apoptotic mediator and an anti-apoptotic regulator. Despite this conclusion, very little research has been done on the contribution of PP2A to apoptotic processes in the heart.

Signalling

Protein phosphatase 2A has been linked to the regulation of several signalling cascades, as exemplified in the p38 MAPK – PP2A – ERK p42/p44 pathway described above. For a review on PP2A and its involvement in signalling see Hunter (1995) and Millward *et al.* (1999). See Chapter 4 for a brief overview of what is known regarding the interactions between PP2A and some of the pro-survival signalling pathways which have been identified in myocardial tissue thanks to studies done on IPC and PostC.

Protein phosphatase 2A therefore presents as a highly abundant protein phosphatase which exerts a significant contribution to serine/threonine phosphatase activity within cells. As such it has been linked to Ca^{2+} homeostasis and the regulation of contractility within the heart and has also been characterized as a regulator and mediator of apoptosis. It exerts many of these effects through its participation in numerous signalling cascades.

General hypothesis and aims

Better and more complete knowledge of the contribution of the phosphatases to myocardial I/R injury and cardioprotection related signalling might reveal possible targets which could be modulated to enhance the efficacy and repeatability of existing cardioprotective interventions by simultaneously modulating both the phosphorylation and dephosphorylation of key signalling role-players.

This study sought to contribute to our basic knowledge and insight regarding the role and importance of protein phosphatase activity in the setting of myocardial ischaemia and reperfusion. We purposefully chose to study I/R injury in a pathology free setting and in the absence of known cardioprotective interventions, since our focus was to describe and explore the most basic

physiological condition. Knowledge gained from these baseline studies can then be applied and tested in more complex situations such as obesity, insulin resistance, IPC, etc.

We hypothesize that PP2A is an important and central participant in the intracellular response of the myocardium to ischaemia and reperfusion, specifically with regards to pro-survival signalling. Should our hypothesis prove to be true, then PP2A is a potential target for the enhancement of current cardioprotective interventions.

To address this hypothesis we concentrated on three aims:

1.) To describe the expression, post-translational modification and intracellular distribution of PP2A as ischaemia progresses, as well as during the clinically important first moments of reperfusion.
2.) To assess if PP2A makes a significant contribution to the development of I/R injury. In order to ascertain this we utilized pharmacological activation and inhibition of PP2A in the context of ischaemia and reperfusion.
3.) To determine the participation of PP2A in pro-survival signalling we sought to determine the signalling-related mechanisms underlying the effects observed in Aim 2.

For the description of PP2A in I/R we utilized both a cell based model of simulated ischaemia/reperfusion, as well as an isolated rat heart model. By comparing the results obtained from these two different models we hoped to generate a more clear picture of how PP2A participates in I/R.

The rest of the aims were addressed using the isolated rat heart exposed to either 35 minutes of regional ischaemia, or 20 minutes of global ischaemia. This allowed us to investigate the effects of PP2A activity modulation in a setting which is more physiological than the cell based model.

CHAPTER 2

Materials and Methods

“Science works by experiments. It watches how things behave. Every scientific statement in the long run, however complicated it looks, really means something like, 'I pointed the telescope to such and such a part of the sky at 2:20 a.m. on January 15th and saw so-and-so,' or, 'I put some of this stuff in a pot and heated it to such-and-such a temperature and it did so-and-so.' Do not think I am saying anything against science: I am only saying what its job is.”

C.S. Lewis
Mere Christianity

Chapter 2 - Materials and Methods

In pursuit of defining the role and importance of PP2A in the setting of myocardial I/R we utilized two fundamentally different experimental models: (1.) A cell culture model; and (2.) an isolated rat heart model. Both these models have advantages and disadvantages and by utilizing both we sought to generate a robust picture of the events surrounding I/R.

For the cell culture experiments we utilized a myoblast cell line of cardiac origin – the H9c2 cell line (Kimes & Brandt, 1976). The advantages of this model lie in its technical and experimental robustness: the cells are relatively easy to culture and maintain, they offer a relatively homogeneous cell population and since they are one of only a few cell lines of cardiac origin they have been used extensively by many cardiac researchers. This model allows for the use of cell-based techniques such as flow cytometry, immunofluorescence microscopy and fluorescence-based enzyme and survival assays. The H9c2 cell line therefore offers a standardized, widely accepted experimental model with a broad application. We therefore utilized the H9c2 cell model as part of our study describing PP2A in the context of I/R. Any cell-model however has the disadvantage that it functions within a supremely unphysiological environment and is therefore only an approximation of what is really happening within a cell, in an organ, in a whole organism. This is especially true of a cell line such as the H9c2 cells which differs from heart tissue in several ways, the most notable being that these cells do not beat; which implies reduced energy demand and intracellular Ca^{2+} flux compared to beating ventricular cells. Also, in contrast to terminally differentiated cardiomyocytes, H9c2 cells are proliferating cardiac progenitor cells. In 1991 Hescheler *et al.* investigated some of the characteristics of this cell line, and although it is true that these cells do not have the same morphology as isolated heart cells, they have a similar surface coat composition, L-type Ca^{2+} current across the plasma membrane, and a G protein pattern similar to striated muscle cells and adult cardiac myocytes. These authors therefore concluded that the H9c2 cell line may be used as a cardiac cell model with regards to transmembrane signal transduction.

The isolated rat heart model however offers more insight into phenomena and mechanisms within a much more physiological setting: an actual beating heart. This experimental model has also been extensively described and utilized by many researchers (for a review on the technique and its applications see Sutherland & Hearse, 2000). Although it obviously also functions within an unphysiological environment (a heart functioning outside the body), it has the experimental advantage that the researcher can simplify the system to which the heart is exposed, relative to the internal environment of the body. This allows for more efficient investigation of an intervention and the

outcomes associated with that intervention. We utilized the isolated working rat heart as the experimental model of choice for the majority of our investigations.

Cell culture

Culture and maintenance

H9c2 cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured at 37ºC in a 40 – 60% humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and 4.5 g/L of glucose (Lonza; BE12-604F) supplemented with 10% fetal bovine serum (FBS) (Highveld Biological) and 1% Penicillin-Streptomycin (10 000 units/mL, Invitrogen; #15140122) mixture. Cell medium was replaced every 2-3 days.

On reaching a confluency of 80-100% cells were passaged in a ratio of one to between two and four. After removal of the DMEM growth medium, cells were washed twice with warm (room temperature) sterile phosphate buffered saline (PBS), whereafter they were incubated in 0.25% Trypsin-EDTA (containing 0.105 mM trypsin and 0.913 mM Na2•EDTA) until the cells detached from the plate surface (approximately 1-4 minutes). The resulting Trypsin-cell mixture was then added to a volume of DMEM growth medium (either equal to, or half of the volume trypsin used). This cell-suspension was then centrifuged at 657xg for 4 minutes. The supernatant was removed and the pellet resuspended in the appropriate volume of growth medium to obtain a density of $3x10^4$ – $6x10^4$ cells/mL. Depending on the type of experiment, cells were seeded in either 35x10 mm or 100x20 mm cell culture dishes (SPL Life Sciences) in a volume of either 2 ml or 10 ml DMEM growth medium respectively.

Simulated ischaemia and reperfusion

As mentioned previously, the H9c2 cell line has been used extensively as a cardiac cell model for various experimental interventions, including hypoxia and simulated ischaemia (Bonavita *et al.,* 2003; Agnetti *et al.*, 2005; Lin *et al.,* 2010). In order to expose the cells to a situation comparable to tissue ischaemia we incubated 80-100% confluent H9c2 cells in a modified Esumi buffer (Esumi *et al.*, 1991) containing (in mM): NaCl 137, KCl 12, Cl_2Mg 0.5, $CaCl_2•2H_2O$ 0.9, HEPES 4, lactate 20 and 2% FBS at a pH of 6.4. This buffer simulates ischaemia with regards to several parameters: an elevated extracellular potassium concentration, low nutrition (reduced FBS and no glucose), low pH (combined with a low pH buffering capacity) and accumulation of toxic waste products (the presence of lactate). Although lactate in itself could potentially directly inhibit the glycolytic pathway (Leite *et al.*, 2007 & 2011) we also experimented with the addition of the glycolytic inhibitor 2-deoxy-glucose (2-DG, 20 mM) (Fuglesteg *et al.,* 2008) and / or the mitochondrial inhibitor sodium dithionate (SDT, 0.5 mM)

(Cumming *et al.,* 1996). In addition to these chemical interventions, incubation of the cells in Esumi-buffer was also combined with exposure of the cells to a hypoxic atmosphere. Initial experiments were done in a cell incubator set at 5% CO_2, 1% O_2 and the balance N_2. Due to technical problems we later replaced this system with a hypoxia incubator chamber (Stemcell technologies) in which we performed the brunt of our experiments. In this hypoxic chamber cells were incubated at 5% CO_2, 0.5% O_2 and the balance N_2. The effects of different durations of exposure (2h, 4h, 6h and 24h) on cell survival were also tested. For a review on work done on simulated ischaemia (SI) and hypoxia, as well as ischaemic preconditioning, in different cell models, see Diaz and Wilson (2006).

Simulated reperfusion simply entailed removal of the ischaemic buffer, washing the cells once with warm PBS where after the cells were returned to the standard 21% O_2 / 5% CO_2 atmosphere and incubated in normal DMEM growth medium.

Detemination of cell viability and cell death

Cell death was determined using flow cytometry based measurement of fluorophores associated with cell death. Two fluorophores were used which allow simultaneous distinction between necrotic and apoptotic cell death: (1.) Propidium Iodide (PI) binds DNA, but is not able to cross the cell membrane. Therefore a positive PI signal will imply that the cell membranes have become permeable, as would be the case during late stage apoptosis or necrosis. (2.) Annexin V (AV) binds phosphatidylserine which is usually only found on the intracellular side of the plasma membrane, however during early apoptosis the plasma membrane loses its asymmetry causing the phosphatidylserine to become accessable for AV binding on the extracellular side. Fluorochrome-labeled AV can therefore be used to identify cells undergoing apoptosis. Both PI and Alexa Fluor® 647 AV were from BioLegend.

Briefly, cells were exposed to SI (as described above), as well as simulated ischaemia / reperfusion (SI/R) for different durations of time (as will be defined in Chapter 3). Following this cells were washed twice with sterile PBS, trypsinized and resuspended in binding buffer (also obtained from BioLegend) at a concentration of approximately $1x10^6$ cells/mL. Of this cell suspension 100 µL (i.e. 100 000 cells) was assayed by the addition of 10 µL PI and 5 µL Alexa Fluor® 647 AV. Following addition of the fluorophores the cell suspension was incubated for 15 minutes in the dark at room temperature. Samples were then measured using the Becton Dickinson FACSCalibur (BD Biosciences), set to acquire between 5000 and 10 000 events – depending on the yield of cells on the day. The resulting fluorescence spectra was analyzed using WinMDI (Windows Multiple Document Interface Flow Cytometry Application, developed at the Scribbs Research Institute and avalaible at

http://facs.scripps.edu/software.html) in order to quantify the percentages of the cell population undergoing apoptosis and / or necrosis.

For each intervention (SI and SI/R) three repetitions were done on three different days. Each repetition consisted of three control cell dishes, while five to six dishes were exposed to the relevant experimental intervention.

Animals

Experimental protocols relating to animal experiments were approved by the Animal Ethics committee of the University of Stellenbosch (Faculty of Medicine and Health Sciences). All animal experiments were done in accordance to the "Guide for the care and use of laboratory animals" published by the US National Institutes of Health (NIH application no 85-23, revised 1985).

Male Wistar rats were given free access to food and water until the time of experimentation. Animals weighing between 200 and 350 gram, were anaesthetized by administration of an intra-abdominal injection of pentobarbital, at a dose of approximately 60 mg/rat.

Isolated rat heart perfusion

Having determined sufficient sedation (as determined by foot pinch test), hearts were rapidly excised and arrested in ice-cold Krebs-Henseleit solution. Hearts were then immediately mounted on the perfusion apparatus by cannulation of the aorta. Once the aorta was secure, hearts were retrogradely perfused using a Krebs-Henseleit bicarbonate buffer (KHB) containing (in mM): NaCl 119; $NaHCO_3$ 24.9; KCl 4.74; KH_2PO_4 1.19; $MgSO_4 \bullet 7H_2O$ 0.6; Na_2SO_4 0.59; $CaCl_2 \bullet 12H_2O$ 1.25 and glucose 10. The perfusion buffer was continuously gassed with a 95% O_2 / 5% CO_2 combination in order to ensure adequate oxygenation of the heart, as well as to maintain the pH of the buffer at 7.4. The perfusion apparatus consisted of double-walled glasware, connected to a circulating waterbath, in order to maintain the heart's temperature at approximately 36.5 °C. Cardiac temperature during experimentation was monitored using a thermal probe inserted into the coronary sinus. Functional parameters such as intra-aortic pressure generated and heart rate were measured by a pressure transducer (Viggo Spectromed) inserted into the aortic perfusion line and connected to a computerized system.

Through the course of experimentation hearts were exposed to three different modes of perfusion:

1.) **Retrograde perfusion (Langendorff perfusion):** Hearts were retrogradely perfused with Krebs-Henseleit buffer at a pressure of 100 cm H_2O. Coronary flow (CF) was measured by timed collection of coronary effluent.

2.) **Work mode:** This required cannulation of the left pulmonary vein (which was done immediately following aortic cannulation). During work mode retrograde perfusion was discontinued, and replaced by the infusion of KHB into the left atrium at a preload of 15 cm H_2O. This inflow of fluid into the left ventricle stimulated ventricular contraction, thereby expelling buffer through the aorta against an afterload of 100 cm H_2O. The volume of buffer pumped by the heart, i.e. the aortic output (AO), was then manually measured and expressed as volume flow per minute.

   Besides aortic output, left ventricular work performance was also used to assess cardiac function. Work performance was calculated using the formula described by Kannengieser *et al.* (1979):

   $0.002222\ (P_{AO} - 11.25)(CO)$

   Where: P_{AO} = aortic pressure

   CO = cardiac output (aortic output + coronary flow rate).

3.) **Induced ischaemia:** We utilised two different forms of ischaemic insult during retrograde perfusion: (1.) Global ischaemia (GI) was applied by the total cessation of retrograde coronary perfusion of the heart, leaving the whole heart ischaemic. The damage conveyed by global ischaemia was assessed by calculating the percentage recovery in functional parameters after 30 minutes reperfusion. Where functional recovery was the main end-point at least five hearts were included in the control group, while a minimum of four hearts were used in the experimental groups. Specific n-values will be reported at the relevant sections in the text. The advantage of this intervention is that the whole heart is exposed to ischaemia and therefore presents as a homogeneous cell population which can then be subjected to biochemical analyses, such as enzyme activity determination or Western blotting.

   In contrast to global ischaemia, (2.) regional ischaemia (RI) was induced by the occlusion of the left coronary descending artery. This was done by using a silk suture to ensnare the proximal part of the left coronary artery. The suture was then closed by using two pieces of interlocking tubing, leaving tissue distal to the occlusion ischaemic (referred to as the area at risk (AAR)), while the

surrounding heart muscle still received adequate perfusion (the viable area (VA)). Reperfusion was initiated by simply opening the two pieces of tubing. Damage associated with this intervention was primarily quantified by measuring the area of necrosis (the infarct size (IFS)) following 60 minutes of reperfusion. This end-point is considered the "gold standard" for assessing the outcome of I/R injury. Function and functional recovery were also recorded.

During both forms of ischaemia cardiac temperature was continuously monitored and maintained at 36.5 °C.

Determination of infarct size

As described in the previous section, regional ischaemia was one of the ischaemic models used in this study. This intervention allows for the determination of the effects of an I/R protocol on tissue viability by measuring the associated infarct size as follows:

At the end of reperfusion the silk snare surrounding the left descending coronary artery (which was used to initiate ischaemia) was carefully re-closed and the heart retrogradely infused with 0.5% Evans Blue dye through the aortic cannula. This served to clearly indicate which area of the heart received normal perfusion throughout the regional ischaemia intervention (the viable area (VA)). The heart was then removed from the perfusion apparatus and frozen (-20 °C) for a period of 1 to 5 days. Following this, the left ventricles (from where the suture was inserted to the apex of the heart) were sliced into approximately 2 mm thick slices. These slices were then stained by incubating the slices in a phosphate buffer (pH 7.4) containing 1% w/v triphenyltetrazolium chloride (TTC) for 15 minutes, at room temperature. TTC reacts with active dehydrogenases in viable tissue to form a brick-red precipitate (Pitts *et al.* 2007). This staining reaction was then terminated by fixing the slices in a 10% v/v formaldehyde solution. Following this procedure, three different coloured-areas should be visible: a dark blue area indicating the viable area, a brick-red area indicating tissue exposed to ischaemia but still viable and a non-stained white area in which all the cell membranes were permeable to intracellular enzymes, such as the dehydrogenases (i.e. a necrotic area).

These area's were quantified by tracing each ventricular slice on a transparency, scanning it into a computer and using the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas, which is available from the internet at http://ddsdx.uthscsa.edu/dig/itdesc.html) to quantify the relevant surface areas as described above.

For the determination of IFS a minimum of six animals were used as controls for each experimental intervention, while hearts from at least six animals were used for pharmacological intervention. In each of the following chapters the precise n-values will be supplied in conjunction with the relevant experiments.

Western Blotting protocol

H9c2 cells: Protein extraction

In order to ensure a sizable protein yield cells were cultured in 100x20 mm cell culture dishes. Similarly to the FACS analysis previously described, for Western blotting experiments three repetitions of the experiments were done on three separate days, with each repetition containing three control cell dishes, as well as five experimental dishes. Following exposure to several simulated ischaemia and/or reperfusion time durations (as will be outlined in the appropriate sections), cells were trypsinized as described before. Following the initial centrifugation step, the supernatant was decanted and the cell pellet resuspended in PBS. Cells were then centrifuged again, the PBS removed and the pellet lyzed by the addition of a detergent-containing lysis buffer in combination with mechanical homogenization. The lysis buffer contained (in mM): Tris-HCl (pH 7.5) 200; EGTA 10; EDTA 100; NaCl 1000; Triton X-100 1%; a cocktail of phosphatase inhibitors: β-glycerophosphate 1, tetrasodiumpyrophosphate 2.5, Na_3VO_4 10; and the protease inhibitors: phenylmethylsulphonylfluoride (PMSF) 100, leupeptin 10 µg/ml and aprotonin 10 µg/ml. Mechanical homogenization was done by adding 0.5 mm diameter zirconium oxide beads (Next Advance, Inc.) to the cell pellet and using a Bullet Blender™ (Next Advance, Inc.) at a setting of 1, for 3 cycles of 1 minute each, interspersed with 5 minutes, at a temperature of 4ºC. Following this, the homogenate was spun down at 1000xg for 15 minutes and the supernatant removed from the bead-containing pellet and kept for protein determination and subsequent Western Blotting.

Isolated rat heart: Collection of tissue samples

For Western blotting analysis of tissue we used a minimum of three hearts as controls (with the exception of the analysis of the effects of FTY720 under baseline conditions in chapter 5, where only two hearts were used), while the different experimental interventions ranged between three and six hearts. The specific n-values will be reported at the appropriate sections in the text. As will be described in more detail later in the text, hearts were freeze-clamped at different timepoints in the perfusion protocol using liquid nitrogen cooled tongs and immediately plunged into liquid nitrogen. These tissue samples were then stored in liquid nitrogen for later analysis.

Isolated rat heart: Protein extraction

Non-fractionated whole cell lysates

Approximately 0.2 gram of frozen tissue samples were pulverized in a stainless steel mortar under liquid nitrogen cooled conditions, where after the tissue samples were immediately transferred to 700-900 µL lysis buffer (same composition as described above for the homogenization of the H9c2 cells). Samples were then homogenized using a SilentCrusher M homogenizer (Heidolph Instruments) set at a speed of 15000 rpm for 2-3 cycles of 5 seconds each. Following this, lysates were left to stand for 20 minutes, followed by 1000 g centrifugation for 15 minutes. The initial inclusion of a detergent (Triton X-100) in the lysis buffer, as well as the 20 minute incubation step led to the degradation of all the organel and cellular membranes, thereby yielding a cellular homogenate containing content from all the cellular compartments. Following centrifugation the supernatant was separated from the pellet and used for further experimentation. Samples were kept at 4 °C at all times.

Following extraction the amount of protein was determined using the Bradford technique (Bradford; 1976). Lysates were then appropriately diluted with lysis buffer in order to ensure that the final samples contained equal amounts of protein. Finally, Laemmli sample buffer was added in a ratio of 1:2, where after the samples were boiled for 5 minutes and stored at -20 °C.

Cell fractionation

The cell fractionation protocol utilized (Figure 2.1) was a differential centrifugation fractionation protocol modified from Williams & Ford (2001) and Sakamoto *et al.* (2000), which has also previously been used in our laboratory (Marais *et al.*, 2005). Although this protocol can only generate crude fractions, it was ideally suited for this study in that it served as a platform for initial and explorative characterization.

As described above, frozen tissue samples were powdered under liquid nitrogen cooled conditions and immediately transferred to lysis buffer. Exactly the same lysis buffer was used as described above, except that it contained *no Triton X-100*. Samples were homogenized using the SilentCrusher M, incubated for 20 minutes on ice and centrifuged for 10 minutes at 1000xg (centrifugation 1). The supernatant was then separated from the pellet and again centrifuged for 60 minutes at 100 000xg. The supernatant following the second centrifugation contained the cytosolic fraction, while the pellet contained the crude membrane fraction, which was resuspended in 300 µL of lysis buffer and homogenised using a Teflon homogenizer. The original pellet (generated in centrifugation 1) was also resuspended in 250 µL lysis buffer supplemented with 1% Triton X-100 and homogenized using a Teflon homogenizer. This homogenate was then incubated on ice for at least 10 minutes, followed by

a second centrifugation at 15 000xg for 30 minutes. The supernatant generated in this spin contained the nuclear extract (see Figure 2.1).

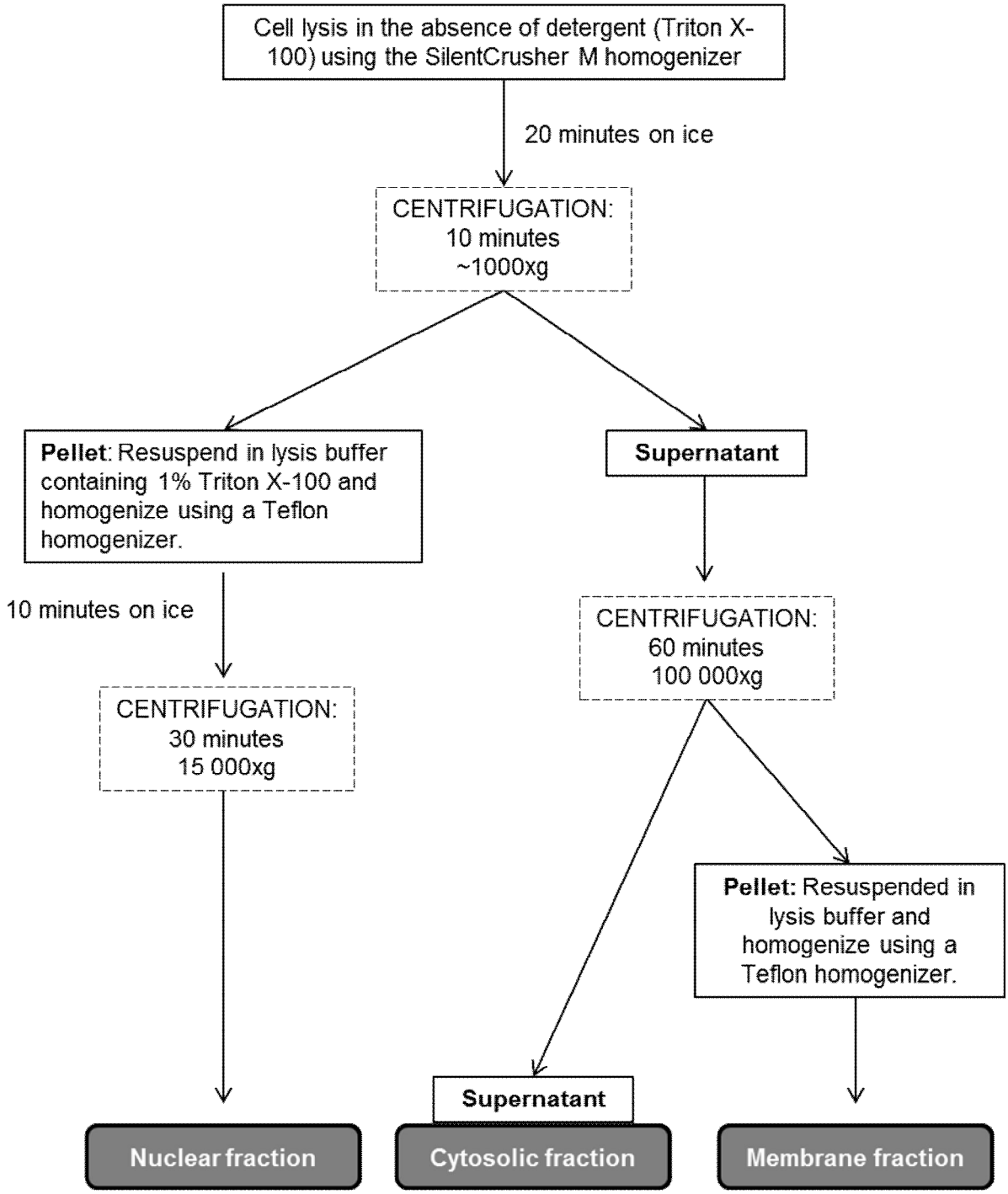


Figure 2.1: Differential centrifugation protocol for the separation of tissue lysates into several different cellular fractions.

As described above for the whole cell lysates, the protein content of each fraction was determined using the Bradford technique, followed by dilution of all samples to a uniform protein concentration to which Laemmli sample buffer was added and the samples boiled for 5 minutes and stored at -20 °C.

Protein separation

Proteins in the lysate samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were first boiled for 5 minutes, followed by brief centrifugation (4-5 minutes), before being loaded in a 4% stacking polyacrylamide gel and separated in a 12% polyacrylamide gel, using the standard Bio-RAD Mini-PROTEAN II System. Running buffer used contained (in mM): Tris 25; glycine 192 and sodium dodecyl sulfate (SDS) 3.5. For each SDS-PAGE experiment, an equal amount of protein per sample was loaded.

Western blotting

After SDS-PAGE separation, proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane (Immobilon™ P, Millipore) using a tank electrotransfer system. For transfer the membrane-gel stack was immersed in transfer buffer containing (in mM): Tris 25; glycine 192 and 20% methanol. The quality of the transfer was regularly assessed by staining the membranes after transfer with the reversible protein stain Ponceau red.

Blocking of the membrane

Following transfer, non-specific protein binding sites on the PVDF membranes were blocked by incubating the membrane in 5% fat-free milk in washing buffer (Tris-buffered saline (TBS)-0.1% Tween 20 solution), under constant agitation, for at least one hour.

Incubation with antibodies

Between blocking and antibody incubation the membranes were thoroughly washed with three changes of TBS-Tween, followed by at least 4 cycles of 5 minutes each washing with larger volumes of TBS-Tween. This washing regime was also utilised between primary and secondary antibody incubation, as well as prior to visualisation.

Following sufficient washing the membranes were incubated with the relevant primary antibody for at least 5 hours or overnight, at 4 °C, under constant agitation. Antibodies were diluted in TBS-Tween with the appropriate dilution factor (1:1000). Antibodies used for this study are listed in table 2.1. All antibodies were raised in rabbit, except nonmethylated PP2A-C which is of murine origin.

Table 2.1: Antibodies used for Western Blotting analyses throughout this study.

Cell Signaling Technology	
Antibody against total protein	**Antibody against a post-translational modification**
PP2A-C (catalytic subunit)	Nonmethylated PP2A-C
PP2A-A (scaffolding subunit)	
PKB/Akt	Phospho-PKB/Akt (Ser473)
GSK3β	Phospho-GSK3β (Ser9)
ERK p42/p44	Phospho-ERK p44/p42 (Thr202/Tyr204)
p38 MAPK	Phospho-p38 MAPK (Thr180/Tyr182)
B-Tubulin	
R & D Systems	
	Phospho-PP2A-C (Tyr307)

This was again followed by a washing cycle, after which the membranes were incubated with appropriate horseradish peroxidase-linked secondary antibody. For most primary antibodies this was ECL™ anti-rabbit immunoglobulin G (from donkey), but for nonmethylated PP2A-C Subunit ECL™ anti-mouse immunoglobulin G, (from sheep) was used. Both secondary antibodies were obtained from Amersham.

Visualisation and analysis

Membranes were covered with ECL™ detection reagents for one minute and then immediately exposed to autoradiography film (Hyperfilm ECL, RPN 2103). Suitable time was allowed for adequate light emmision to be generated by the luminescence reaction between the detection reagents and the conjugated horseradish peroxidase.

Densitometric analyses of the membranes were done using UN-SCAN-IT™ version 5.1, Silkscience. In order to take inter-membrane variability into account, and for comparison purposes, all experimental groups were expressed as a ratio of the average of the relevant control hearts. In the case of post-translational modifications (i.e. phosphorylation and nonmethylation) the ratio between the modified form and the total was calculated using the original pixel values. The calculated ratio was then normalized to the average of the control ratios. All ratios and normalized data were expressed in arbitrary units (AU).

Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was employed to determine possible substrates of the catalytic subunit of PP2A (PP2A-C). Four hearts were included in the control group, as well as in each of the experimental groups. Tissue samples collected from isolated rat hearts at the end of sustained ischaemia as well as at the onset of reperfusion were pulverized under liquid nitrogen conditions and added to 850 µL of lysis buffer with a similar composition to the one used for Western Blotting but with two crucial differences: (1.) the concentration of the detergent (Triton X-100) was reduced from 1% to 0.1% in order to conserve protein-protein interactions; and (2.) the serine/threonine protein phosphatase inhibitors β-glycerophosphate and tetrasodiumpyrophosphate were left out to avoid any chemical interference between PP2A and its substrates. Samples were then homogenized using the Silent Crusher M and incubated on ice for ~2 hours, where after they were centrifuged for 15 minutes at 1000xg and the supernatant collected for Bradford protein determination. Approximately 2.5 mg of protein was incubated with 10 µg of a goat poly-clonal antibody against PP2A-Cα/β (Santa Cruz, sc-6110) for 3-4 hours, at 4ºC, under constant rotary agitation. A 1:1 mixture (final volume: 60 µL) of Protein A-Sepharose 4B (SIGMA, P9424) and Protein G-Sepharose 4B (SIGMA, P3296) was then added to the lysate and incubated overnight at 4ºC, under constant rotary agitation.

The next morning the beads were recovered by centrifugation at 1000xg for 30 seconds. The beads were washed once with sterile PBS, centifuged for 30 seconds at 1000xg and the bound proteins eluated by boiling the beads in 40 µL SDS-Laemmli buffer. This final protein sample was then separated using SDS-PAGE and proteins visualized using standard Western Blotting techniques as described above.

Although equal amounts of protein were subjected to the immunoprecipitation protocol we could not be sure that equal amounts of protein were loaded on to the SDS-PAGE gels. This is obviously also a fundamental experimental consequence: if the experimental condition led to an increase in the interactions between PP2A and other cellular proteins, more protein would be co-immunoprecipiated with PP2A-C. The final densitometry results were therefore expressed relative to the signal generated by the primary target of the immunoprecipitation, namely PP2A-C, and reported in Arbitrary Units (AU).

Phosphatase activity assay

Measuring the activity of PP2A in heart tissue proved to be very challenging. We initially utilized a method based on the generation and measurement of free phosphates in tissue homogenates. This serine/threonine phosphatase assay system (Promega, V2460) utilized a phosphopeptide,

RRA(pT)VA, which is a substrate for several of the serine/threonine protein phosphatases (PP2A, PP2B and PP2C). Briefly: free phosphates first had to be cleared from tissue lysates using Sephadex® G-25 spin columns. The phosphate free lysate was then incubated with the substrate, whereafter free phosphate generation was measured using a Molybdate reaction mixture which generates a colourimetric signal at 600-630 nm in the presence of free phosphates. In order to distinguish the activity of PP2A from the other phosphatases, an inhibitor of PP2A (okadaic acid (OA)), had to be added to a separate set of lysates. The OA-sensitive signal was then defined as PP2A dependent. Although this method has been used with success by others (Tikh *et al.*, 2008), we could only measure PP2A in a sample of purified PP2A-C while failing to measure any differences between our experimental groups (hearts perfused with an inhibitor or a possible activator of PP2A, as will be explained in a future chapter), possibly due to a persistently high free phosphate background.

We therefore switched to another, possibly more sensitive, approach based on the protein phosphatase mediated generation of a fluorescent species. There are a number of compounds that can serve as substrates for protein phosphatases and in the process generate fluorescent products which can be measured as a readout of phosphatase activity. One such substrate is 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) which can be hydrolyzed by phoshatases to a more fluorescent species: 6,8-difluoro-4-methylumbelliferone (DiFMU). DiFMUP offers a sensitive tool for the continuous measurement of phosphatase activity within a broad pH range. We utilized the EnzChek® Phosphatase Assay kit from Molecular Probes and modified the protocol according to an article by Wegner *et al.* published in *Methods in Molecular Biology* (2007) in which they described a method to measure PP2A based on the same principle.

Lysates were prepared, as already described, from heart tissue (control n=2, experimental intervention n=4) in a lysis buffer containing the following (in mM): Tris – HCl (pH 7.5) 200, EGTA 8, EDTA 8, NaCl 1000, PMSF 100, Triton X-100 1%; leupeptin 10 µg/ml and aprotonin 10 µg/ml. Following Bradford protein determination samples were immunoprecipitated using a similar protocol as already described: 1500 µg of protein was incubated with 6 µg of anti-PP2A-Cα/β (Santa Cruz, sc-6110) as well as 75 µL of protein A/G bead slurry (Protein A/G PLUS-Agarose Immunoprecipitation Reagent, Santa Cruz, sc-2003) overnight, under rotational agitation at 4ºC. Beads were recovered and washed twice with PBS and resuspended in 145 µL of 200 mM Tris (pH 7.5) buffer. Two duplicate sets of 35 µL each of sample were then added to a black 96-well plate (SPL Life Sciences). To one set 10 µL OA was added at a PP2A-specific concentration of 10 nM, while the other set received an equivalent volume of Tris buffer. This was necessary to specifically distinguish and quantify the contribution of PP2A to the signal generated. Additionally 5 mg/mL bovine serum albumin (BSA) was also added to the reaction

mixture. Finally 50 µL DiFMUP (Molecular Probes) at a working concentration of 100 µM (200 µM stock) was injected into each sample. Fluorescence measurements were performed on a FLUOstar Omega multi-mode platereader at an excitation / emission wavelength of 355 / 460 nm, approximately every 5 minutes after addition of the substrate for a period of 40 minutes, with additional shaking of the plate before each cycle. The whole reaction was allowed to proceed at room temperature.

The signal measured was corrected for background signal by substracting the signal generated from an appropriate sample void of beads and protein. The PP2A signal was then calculated by substracting the signal generated in the presence of OA from the signal measured in the absence of OA. All values were then normalized to the average of the control group.

Calculation of PP2A activity measurements:

$$\frac{[(\text{Sample} - \text{OA}) - (\text{blank} - \text{OA})] \; - \; [(\text{Sample} + \text{OA}) - (\text{blank} + \text{OA})]}{\text{Average of } [(\text{Control} - \text{OA}) - (\text{blank} - \text{OA})]}$$

Statistical analysis

Results were processed using GraphPad Prism statistical software (versions 5 and 6). All results are presented as average ± standard error of the mean (SEM). Where only two groups were directly compared to each other an unpaired T-test was used to assess the difference. More than one group was compared using an one-way analysis of variance (ANOVA). When all groups were compared with each other, the Bonferroni correction was applied, but when all experimental groups were compared to the control alone, the Dunnett *post hoc* test was utilised. P-values of less than 0.05 were considered significant.

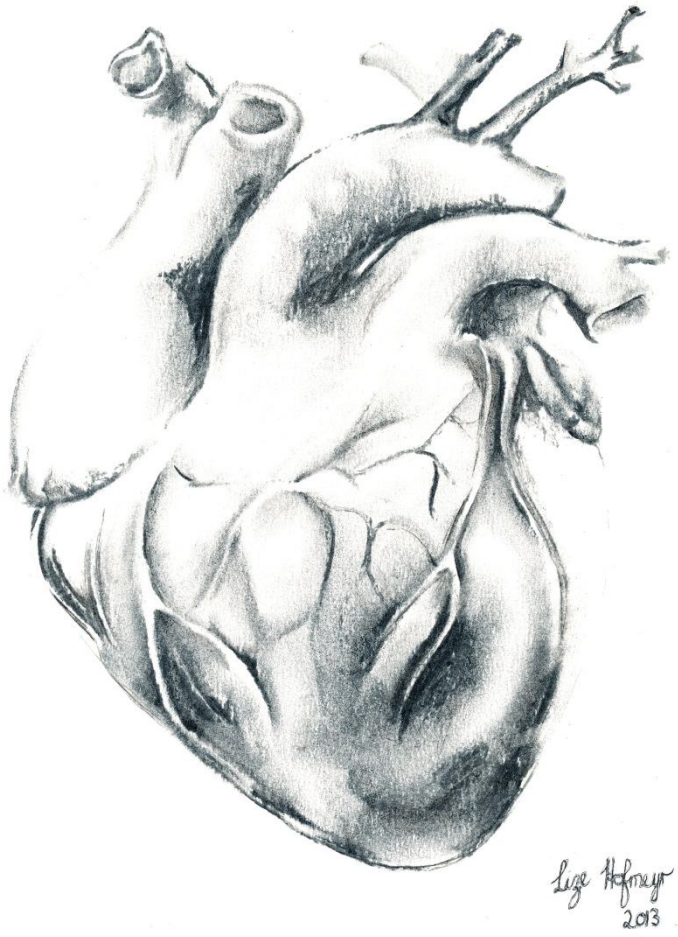
CHAPTER 3

Characterization of protein phosphatase 2A (PP2A) during myocardial ischaemia and reperfusion

"We actually find hidden regularities within the complex variety of a system's behavior."

-Ian Malcom

Micheal Crichton
Jurassic Park



Chapter 3

Characterization of protein phosphatase 2A (PP2A) during myocardial ischaemia and reperfusion

Introduction

Protein phosphatase 2A (PP2A) is a major determinant of the largest portion of protein phosphorylation in mammalian cells, namely serine and threonine phosphorylation (Oliver & Shenolikar, 1998; Olsen *et al.*, 2006). PP2A is a member of the protein serine/threonine phosphatase (PSP) superfamily and is classified as a phosphoprotein phosphatase (PPP), along with other phosphatases such as protein phosphatase 1, protein phosphatase 2B and the lesser known protein phosphatases 4, 5, 6 and 7 (Shi, 2009). Although it is a serine/threonine phosphatase based on the structure of its catalytic site, it would actually be more accurate to think of PP2A as a dual specificity phosphatase, since under certain conditions (as will be discussed later in this section) it demonstrates tyrosyl phosphatase activity.

For a long time the phosphatases were dismissed as passive dephosphorylators of signalling pathways, while the kinases were considered the dynamic regulators of signalling, especially when taking into consideration that there are considerably fewer protein phosphatases than kinases coded for in the human genome (Shi, 2009). It was therefore assumed that the phosphatases were regulated to a minimum extent. Recent research has revealed that nothing is further from the truth. This section will introduce the reader to what is currently known regarding the regulation of PP2A in the cell.

Molecular structure

The key to PP2A's ability to dephosphorylate such a large number and wide diversity of substrates, as it does, lies in its modular structure.

The catalytic subunit (PP2A-C)

The active component of PP2A is its catalytic subunit (PP2A-C), a globular protein of ~36 kDa in size, containing two manganese ions at its catalytic site (Xing *et al.*, 2006) which is ubiquitously expressed in all tissues (Janssens &Goris, 2001). Its structure is remarkably well conserved between species (Janssens & Goris, 2001) indicating its importance in the molecular processes of life. Two isofoms of PP2A-C have been identified: α and β. The α isoform is the most abundantly expressed of the two (Janssens & Goris, 2001) and it seems that their functions are not redundant. This was shown by Götz and colleagues (1998 & 2000) who generated a PP2A-Cα knock-out

embryonically lethal mouse model. Their model implicated a role for PP2A-Cα during embryonic development, which could not be salvaged by a likely upregulation of PP2A-Cβ. One of the reasons for this could be the differential cellular distribution of these two isoforms in the embryonic cells. As recently as 2012 an alternative enzymatically inactive spliced isoform of PP2A-Cα was discovered (Migueleti *et* al.). However, the physiological importance of this isoform is unknown, since its generation seems to be dependent on the incubation conditions that *ex vivo* cells are exposed to.

Irrespective of the isoform, PP2A-C has a very broad substrate recognition ability, which poses a potential threat to any phospho-serine, or -threonine residues in the cell. The task of modulating and regulating PP2A-C activity lies with the array of proteins that associate with it.

The scaffold subunit (PP2A-A)

The most important of these is the ubiquitously expressed scaffold subunit (PP2A-A). This scaffold is an elongated structure consisting of 15 HEAT (huntingtin-elongation-A subunit-TOR) repeats, each containing two anti-parallel α-helices. This structure is flexible and allows PP2A-A to bind to PP2A-C, as well as to other proteins. Its association with PP2A-C necessitates a pronounced change in its conformation which might influence both the association of other proteins with the phosphatase complex, as well as the phosphatase activity of PP2A-C (Xing *et al.*, 2006). The dimer formed when PP2A-C and –A interact is also known as the core enzyme. Although PP2A-A was initially considered as only a scaffold, it has recently been implicated in contributing to the substrate specificity of PP2A (Zhou *et al.*, 2003). Just like PP2A-C, there are two scaffold isoforms: PP2A-Aα and PP2A-Aβ. As is the case with PP2A-C, the alpha isoform is much more abundant than the beta isoform. Interestingly, there are differences in the binding affinities of the two isoforms for different binding-partners, as well as in tissue distribution. Of the two, the alpha subunit shows a greater affinity for most binding-partners as well as a more homogenous expression in all tissues (Zhou *et al.*, 2003).

The regulatory subunits (B subunits)

Additionally to the scaffold, a third subunit can also associate with the C subunit, namely the regulatory B subunit. Simply calling it the B subunit is, however, a gross simplification. In reality the “regulatory subunit” refers to a vast group of proteins consisting of four unrelated families: B (also known as R2, or PR55), B’’ (R3 or PR72), B’ (R5, B56 or PR61) and B’’’ (PR93/PR110). Each of these families contain multiple isoforms: Bα – δ, B’’α – γ, B’α – ε. There are therefore approximately 15 regulatory subunits. Both PP2A-C isoforms bind with equal affinity to the A and B subunits, while PP2A-Aβ has a lower affinity for both PP2A-C and B (Zhou *et al.*, 2003). The implication of this is that any of these different C, A and B isoforms can theoretically associate with each other to generate at least 75 different enzymatically active versions of PP2A. Unlike the C

and A subunits, the regulatory subunits are not expressed in all tissues, in fact they show a definite tissue and subcellular distribution. Therefore, it is thought that the regulatory subunit acts to "target" the core AC enzyme to specific intracellular locations thereby bringing it into proximity with its substrate(s) (Janssens & Goris, 2001; Silverstein *et al.*, 2010; Sents *et al.*, 2013).

Addition of a regulatory subunit gives rise to the trimeric or so-called holoenzyme. Studies of the crystal structure of the PP2A holoenzyme has revealed that the B subunit binds to both the A and C subunits, although it forms a relatively weak bond with the scaffold. The addition of B subunit to the dimer influences the substrate specificity of the C subunit by changing the accessibility of the catalytic site for different substrates (Xu *et al.*, 2006; Cho *et al.*, 2007). In 1991 Kamibayashi and colleagues showed that dissociation of the B subunit leads to an increase in both the V_{max} and K_m-values of PP2A, indicating that the B subunit indeed influences substrate affinity, as well as – perhaps unexpectedly – also the catalytic ability of PP2A.

Although I shall briefly touch on some of the research concerning the dynamics of the regulatory subunit, it is not the purpose of this chapter to give an exhaustive account of the distribution and substrate-targeting patterns of the regulatory subunits. For this the reader is referred to Silverstein *et al.*, 2010.

B'' / PR72 / R3 isoform

The rat form of the B'' / PR72 subunit was described in 1997 (Nagase *et al.*) and was detected in all tissues including the heart with an intracellular distribution targeted to the cytosol, microsomes and mitochondria. This intracellular distribution was later expanded to include the nucleus (Kremmer, 1997). The targeting capability of the regulatory B subunits is nicely exemplified by the ability of PR130 (a B'' / PR72 isoform) to target PP2A to RyR2 in the heart (Marx *et al.*, 2001). Interestingly Nagase and colleagues (1997) noted that B'' could also be phosphorylated by PKA, thereby leading to an increased activity of PP2A against some, but not all, of its substrates (Usui *et al.*, 1998). This illustrates the potential for the regulatory B subunits to be targeted and modified by cellular processes in order to modify the activity or substrate specificity of PP2A.

B' / PR61 / B56 / R5 isoform

The B'/PR65 (B56) subunit shows a relatively high abundance in cardiac tissue (Kamibayashi *et al.*, 1994; Tehrani *et al.*, 1996; Gigena *et al.*, 2005). A look at this protein reveals the complexity of PP2A regulation. Different isoforms are associated with different cellular locations: α, β and ε are primarily found in the cytoplasm; γ1 and γ3 are targeted to the nucleus; while δ can be found in both the cytoplasm and nucleus, depending on the cell cycle (Tehrani *et al.*, 1996; McCright *et al.,* 1996; Ito *et al.*, 2000; Gigena *et al.*, 2005; Zhou *et al.*, 2007). In the heart it was found that B'α specifically targets PP2A to the M-line and Z-disc of the sarcomere (Bhasin *et al.*, 2007). This is a

dynamic arrangement since β-adrenergic stimulation led to a redistribution of B'α away from these structures (Yin *et al.*, 2010). The targeting ability of the regulatory subunits is not only limited to an area in the cell, but also to specific substrates, an example of this is B' containing holoenzymes that have been found to be involved in the PP2A-mediated dephosphorylation of ERK p42/p44 (Letourneux *et al.*, 2006) and PKB/Akt (Rocher *et al.*, 2007). In an intriguing application of proteomics Zhou *et al*. (2007) identified the binding-partners of specifically B56γ1 from heart tissue as proteins involved in Ca^{2+} handling, as well as nuclear proteins.

The B' subunit is a target of multiple forms of regulation. Most of the isoforms are phosphoproteins which can be phosphorylated. In this this regard PKA has been implicated in the phosphorylation of B'δ, thereby increasing the activity of PP2A, without favouring the dissociation of B' from the holoenzyme (Ahn *et al.*, 2007; Dodge-Kafka *et al.*, 2010). A model of stress in cultured myocytes revealed that the protein levels of only B'α (and not B'γ1) is downregulated in a c-Jun NH2 - terminal kinase (JNK)-dependent fashion.

The importance of the regulatory subunits in also determining the activity of PP2A-C is illustrated in a study by Ito and co-workers (2000). They found that a mutation of B'γ1 in melanoma cells was associated with a reduction in PP2A activity aimed at paxillin, in the absence of a change in the *affinity* of the PP2A-B'γ1 holoenzyme to paxillin.

B / PR55 / R2

In 1994 Kamibayashi *et al.* succeeded in isolating and characterizing a B subunit from cardiac tissue, which they identified as Bβ. Later Strack and colleagues (1999) also isolated Bδ in the heart, as well as confirmed the presence of Bβ. Accentuating the differences between isoforms, Kamibayashi *et al.* (1994) used an insect cell model to express and compare different holoenzymes and found that although Bα and Bβ targeted similar substrates, their response (in terms of activity) to endogenous modulators differed.

Core enzyme versus holoenzyme

Initially it was thought that the dimer core enzyme was not physiologically relevant and only an artefact of the PP2A isolation process. Kremmer and colleagues however proved this wrong and showed that almost a third of PP2A in the cell is actually in the core complex assembly (1997). It could be argued that this constitutes an intracellular pool of enzyme which can be recruited to rapidly generate the holoenzyme. It is however interesting that the core enzyme can recognize substrates which differ from the holoenzyme's substrate selection (Kremmer *et al.*, 1997; Reudiger *et al.*, 1997). The ratio between core enzyme and holoenzyme also seems to be important, since a mutation in the A subunit which favours the formation of the dimeric core enzyme above the

trimeric holoenzyme was associated with the development of a dilated cardiomyopathy phenotype (Brewis *et al.*, 2000).

In conclusion, PP2A is an enzyme which recruits several functional subunits in different combinations, thereby modulating its cellular location, activity and substrate specificity. The effects of the different subunits on some of these parameters were investigated by Price and colleagues (2000). They reported that addition of the A subunit to PP2A-C led to a substantial reduction in the reaction rate and K_m-values of the catalytic subunit. Recruiting a Bα subunit initiated even more pronounced effects: a further reduction in K_m, combined with changes in kinetics and pH dependence.

The question that now arises pertains to the mechanism by which PP2A-C manages to recruit the different B subunits, or otherwise stated: how does PP2A-C allow itself to be recruited by different B subunits?

Regulation of the interaction between PP2A-C and the regulatory subunits

The answer to this question is focussed on a single portion of the catalytic enzyme, namely the carboxylic tail and the posttranslational modification of three of the amino acid residues in this tail.

Phosphorylation at Tyr307 and Thr304

In 1992 Chen and colleagues showed that the catalytic subunit of PP2A can be phosphorylated on its C-terminal tail at tyrosine residue 307 thereby reducing its activity. In this original study they showed that a number of nonreceptor tyrosine kinases and receptor tyrosine kinases, including insulin and epidermal growth factor receptors, can phosphorylate PP2A-C. At the same time, Guo and co-workers (1993) purified and characterized a novel serine/threonine kinase with an autophosphorylation capacity that has the ability to phosphorylate PP2A on a threonine residue, thereby also inhibiting the enzyme. Possibly the most intriguing result from these studies was the observation that the pharmacological inhibition of PP2A-C lead to an accumulative increase in the level of phosphorylation (both on tyr307, as well as the threonine residue), indicating that PP2A-C has the ability to dephosphorylate itself. Autodephosphorylation ensures that the inactivation of PP2A remains transient.

Additionally to its implications for enzyme activity, phosphorylation of both tyr307 and thr304 in the C-terminal tail has been implicated in the selection and recruitment of different B subunits. This could either be because the phosphorylated form of these residues might obstruct the physical association between the regulatory subunit and PP2A-C, or in the case of tyr307, its phosphorylation impedes the addition of a methyl group on leucine residue 309 (Ogris *et al.*, 1997;

Cho *et al.*, 2006; Nunbhakdi-Craig *et al.*, 2007; Longin *et al.*, 2007). This brings us to another important regulatory posttranslational modification of PP2A-C: methylation.

Methylation of Leu309

Leucine 309 is also located in the C-terminal tail, in fact it is the last amino acid residue of PP2A-C, and it has the potential to be methylated. The crystal structure of the PP2A holoenzyme (Cho *et* al., 2007) revealed that the methylation status of the C-terminal tail influences its interaction with the A subunit, thereby modulating the availability of a binding site for the regulatory subunits. Several studies utilizing mutational approaches found that the methylation of leu309 is indeed a determining factor in the recruitment of regulatory subunits, in that some subunits are only found associated with the methylated form of PP2A-C, while the binding of other B subunits seem to be independent of methylation (Bryant *et al.*, 1999; Schild *et al.*, 2006; Nunbhakdi-Craig *et al.*, 2007; Longin *et al.*, 2007). This regulatory mechanism is only aimed at selecting the regulatory subunit and it does not influence the formation of the PP2A-AC core dimer enzyme (Schild *et al.*, 2006; Longin *et al.*, 2007). With regards to this, it is interesting to note that a larger fraction of holoenzyme trimers are methylated, while the dimer core enzyme seems to mostly be nonmethylated (Bryant *et al.*, 1999; Longin *et al.*, 2004). Of the regulatory subunits which have been studied it seems that the binding of B'' (PR72) and B''' (PR93/PR110) is independent of methylation, while the recruitment of B is more (although not absolutely) dependent on methylation. Interestingly Ikehara *et al.* (2007) found that the methylation status of PP2A had no effect on trimer assembly with the B (PR55) subunit. Their observations can probably be ascribed to the fact that they studied a specific subunit isoform in a specific *milieu* which is not that sensitive to methylation. The dependence of B' (B56 / PR61) on methylation seems to be uncertain (Bryant *et al.*, 1999; Yu *et al.*, 2001; Nunbhakdi-Craig *et al.*, 2007; Longin *et al*., 2004). The impact of methylation on regulatory subunit recruitment is also summarized in Table 3.1.

Unlike tyr307 phosphorylation, the methylation of PP2A-C does not seem to influence its activity *per se*, although by influencing B subunit association it will necessarily influence catalytic activity and substrate specificity (Bryant *et al.*, 1999; Schild *et al.*, 2006; Ikehara *et al.*, 2007).

As described previously, the regulatory subunit in a holoenzyme is essential in determining the cellular location of PP2A. It therefore follows that the methylation of PP2A is important in determining the cellular distribution of the enzyme. In this regard Longin and colleagues (2008) reported a specific cellular distribution of PP2A methylation with higher levels of nonmethylated PP2A in the nucleus and a higher proportion of methylated PP2A in the cytoplasm. The functional implications of PP2A-C methylation has recently fallen under the spotlight. It has been shown that adenosine A1 receptor stimulation of isolated rat hearts induces the translocation of PP2A from the cytosol to the particulate fraction concurrent with an increase in the level of methylation, all in a G_i-

dependent manner (Liu & Hofmann, 2002). Similarly exposure of isolated rat cardiomyocytes to hydrogen peroxide (H_2O_2) led to an increase in PP2A-C methylation, as well as a shift in PP2A activity from a Triton-soluble cytosolic to the myofilament-particulate fraction. This redistribution was however blocked by the inhibition of methylation (Deshmukh *et al.*, 2007). It is noteworthy that these authors also measured some adrenergic-stimulated translocation of B'α from the cytosol to the particulate fraction. This is quite possible, since B'α has been found in both these fractions (Gigena *et al*., 2005; Yin *et al.*, 2010), however their data indicate that the whole holoenzyme translocates in a methylation-dependent manner – in contrast to the existing theory that the dimer is shuttled between regulatory subunits. Their findings are possibly related to alternative splice forms of the B'α isoform, or the involvement of even more intricate and as yet unknown mechanisms. Regardless the precise mechanisms at work, the methylation status of PP2A-C is important in determining the cellular distribution of the enzyme and presents a mechanism of rapidly translocating PP2A.

Table 3.1. The influence of leu309 methylation of the PP2A-C subunit on regulatory subunit association. The specific isoform which was measured by each study is indicated. Three groups are distinguished: "Methylation dependent" absolutely requires leu309 methylation for holoenzyme assembly; "Nonmethylation dependent" is defined as regulatory subunits which prefer to bind to nonmethylated PP2A-C; and "Methylation indifferent" are subunits which seem to bind with almost equal ease to both the nonmethylated and methylated forms of the enzyme.

Regulatory subunit	**Methylation dependent**	**Nonmethylation dependent**	**Methylation indifferent**
B (R2 or PR55)	α – Schild *et al.* 2006 α, β1 – Longin *et al.*, 2007 α – Bryant *et al.*, 1999 α – Nunbhakdi-Craig *et al.* α – Yu *et al*., 2001 α – Lee *et al.*, 2007		α – Ikehara *et al.*, 2007 PR55 – Longin *et al.*, 2004
B' (R5, B56 or PR61)	ε – Schild *et al.* 2006 α, δ – Nunbhakdi-Craig *et al.*	γ – Schild *et al.* 2006	α, β1, δ1, ε – Longin *et al.*, 2007
B'' (R3 or PR72)	PR72 – Longin *et al.*, 2004	β – Schild *et al.* 2006 β – Nunbhakdi-Craig *et al.* (significance not reported)	PR70, PR72 – Longin *et al.*, 2007
B''' (PR93/PR110)		Striatin, SG2NA – Yu *et al.*, 2001	

In summary, the dynamics of regulatory subunit association with PP2A-C – with consequential changes in cell distribution, enzyme activity and substrate specificity – is determined to a large extent by tyrosine and threonine phosphorylation, as well as leucine methylation. Several tyrosine kinases have been implicated in the phosphorylation of tyr307, and at least one serine/threonine kinase has possibly been linked to thr308 phosphorylation. The level of methylation however also

requires rapid and dynamic regulation, accomplished by two enzymes: leucine carboxyl methyltransferase (LCMT-1) and protein phosphatase methylesterase (PME-1).

Leucine carboxyl methyltransferase (LCMT-1)

Recently Lee *et al*. (2007) confirmed that LCMT-1 is the mammalian enzyme responsible for methylating PP2A-C. They went on to confirm the importance of PP2A methylation by showing that silencing LCMT-1 in HeLa cells blocked the assembly of Bα into the holoenzyme which eventually induced apoptosis. Longin and colleagues (2008) investigated the cellular distribution pattern of PP2A methylation and found that LCMT-1 is located in both the nucleus and cytoplasm; in conjunction with a cytoplasmic distribution of methylated PP2A. Intriguingly LCMT-1 favours methylating the active catalytic subunit. This would constitute a mechanism by which PP2A-C is incorporated into a holoenzyme immediately after its activation (Stanevich *et al*., 2011). By modulating PP2A assembly, LCMT-1 has also been implicated in cell signalling. Jackson and co-workers (2012) found that stimulating a reduction in PP2A methylation (by amongst others LCMT-1 knockdown) led to an increase in the activity of PKB/Akt and p70/p85 S6 kinase (S6K), thereby implicating LCMT-1 as a negative regulator of PKB/Akt.

Protein phosphatase methylesterase (PME-1)

Protein phosphatase methylesterase (PME-1) was first identified in 1999 (Ogris *et al*.) and has been confirmed as the primary enzyme responsible for demethylating PP2A (Ortega *et al.*, 2008; Puustinen *et al.*, 2009). It is chiefly located in the nucleus, corresponding with the observation that nuclear PP2A is mostly demethylated (Longin *et al.*, 2008). As is the case for LCMT-1, complete loss of PME-1 is lethal, signifying its importance (Ortega-Gutiérrez *et al.*, 2008).

Interestingly, the activity of both LCMT-1 and PME-1 is reduced in the presence of okadaic acid (Stanevich *et al.*, 2011; Ogris *et al*., 1999). Since okadaic acid binds to the active-site pocket of PP2A-C (Xing *et al.*, 2006) this suggests that LCMT-1 and PME-1 also recognize and associate with the actual catalytic site of PP2A-C. For PME-1 this was recently proven by crystal structure studies (Xing *et al.*, 2008). Intriguingly binding of PME-1 to PP2A-C activates PME-1, and seems to inactivate PP2A-C (Xing *et al.*, 2008). In this sense PME-1 ceases to function as only a catalyst of demethylation, but also acts as a protein exerting a direct effect on PP2A by binding to it.

Binding partners which regulate PP2A

PME-1: safekeeping an inactive pool of PP2A

The original cloning of PME-1 was made possible by the fact that PME-1 was found to bind to a mutational inactive form of PP2A (Ogris *et al.*, 1999). Likewise Longin and colleagues (2004) found PME-1 to be preferentially associated with inactive PP2A, coincidentally mostly the dimeric core

enzyme. These authors speculated that PME-1 might therefore be involved in the maintenance of an inactive intracellular pool of PP2A, possibly in the nucleus (Longin *et al.*, 2008). Although this is a compelling theory, several pieces of data in the literature argue against it: 1.) Ortega-Gutiérrez *et al*. (2009) reported that loss of PME-1 was lethal, implying that demethylated PP2A must be important for survival. They also found that PP2A activity seemed to be negatively influenced by the loss of PME-1, not what you would expect if PME-1 is always linked to inactive PP2A; 2.) It has been reported by several researchers that some of the regulatory subunits bind easily to nonmethylated PP2A. This beckons the question; why would a regulatory subunit bind an inactive form of the enzyme?; 3.) In the original paper concerning the existence of the core enzyme in cells, Kremmer *et al.* (1997) reported that the core enzyme presented with a different substrate specificity than the holoenzyme. This implies that the core enzyme could be active inside the cell, and not merely serve as an inactive pool.

No doubt, the precise mechanisms and significance behind the effects of PME-1 and the methylation balance of PP2A-C still awaits further elucidation. If there is however a pool of inactive PP2A in the cell, the next question would be how the cell will access this pool? The answer to this lies with another very interesting protein which can interact with, and regulate PP2A; namely phosphotyrosyl phosphatase activator (PTPA).

The activator of PP2A: PTPA

It was mentioned earlier in this text that PP2A has the ability to autodephosphorylate its inhibitory phosphorylation site. Although the data indicates that this is indeed the case (Chen *et al.*, 1992), it is problematic since the relevant phosphorylated residue is a tyrosine residue, while PP2A is supposed to be a serine/threonine phosphatase.

Already in 1983 it was realized that PP2A also exhibits tyrosine phosphatase activity, although this PTP component contributes less than its serine/threonine phosphatase activity to the total protein phosphatase activity of PP2A (Chernoff *et al.*, 1983). A comparison of the PTP activity of PP2A with a genuine PTP revealed that PP2A is more limited in the substrates it targets, implying that it must have a physiological role (Agostinis *et al.*, 1996).

In 1990 it was however realized that this PTP activity of PP2A was induced, or activated, by an activator protein which was called phosphotyrosyl phosphatase activator (PTPA). This activator seems to preferentially interact with the dimeric core enzyme in order to stimulate the PTP activity of PP2A to such a degree that it visibly contributes to the total cellular tyrosine phosphatase activity (Cayla *et al.*, 1990). This led to the relatively recent proposal that PTPA drives a shift in the substrate specificity of PP2A (Chao *et* al., 2006). Further characterization of PTPA (Van Hoof *et al.*, 1994) revealed that it is present in all tissues, located in the cytosolic fraction and present in

concentrations in the same range as PP2A, implying that the interaction between PTPA and PP2A must be important. A fascinating aspect of the interaction of PTPA with PP2A is that it requires the presence of Mg^{2+} and ATP and is associated with a low degree of ATPase activity, which can be inhibited by OA. This immediately leads one to suppose that some kind of kinase activity is involved, yet there is no evidence of kinase activity being present (Cayla *et al.*, 1990; Van Hoof *et al.*, 1994; Chao *et al.*, 2006).

A decade after the first descriptions of PTPA, Fellner *et al.* (2003) made the observation, using yeast homologs of PTPA, that it is not only involved in the activation of a PTP activity in PP2A, but it is actually also important for the functioning of the serine/threonine phosphatase activity of PP2A. Deletion of the yeast PTPA homologue was associated with changes in the conformation of PP2A-C which led to shifts in substrate specificity and a reduction in protein stability. This illustrated that PTPA could somehow modify the actual structure of PP2A-C, thereby influencing all aspects of PP2A activity. Linking with this, Longin and co-workers (2004) found that PTPA could re-activate the serine/threonine phosphatase activity of an inactive pool of PP2A bound to PME-1. This pool consisted mostly of core enzyme and although the activation process required ATP hydrolysis, it did not seem to be transient nor did it require continuous energy input. The affinity of PME-1 for active PP2A is lower than for the inactive form, also ensuring that the newly activated PP2A could not easily be "trapped" back in storage as an inactive enzyme. These authors therefore suggested that the name of PTPA be changed to "**p**hosphatase **t**wo A **p**hosphatase **a**ctivator". These studies therefore clearly implicated PTPA as a major contributor to PP2A activity, but the mechanism how it accomplishes this was still a mystery.

Two years later in 2006, Jordans *et al*. classified PTPA as a peptidyl-prolyl *cis/trans*-isomerase and linked this activity of PTPA to its activation function. They proposed that PTPA targets and isomerizes prolyl 190 in PP2A-C, thereby altering the conformation of PP2A-C from an inactive to an active form. This might explain the requirement for Mg^{2+} and ATP, since it is plausible that inducing a conformation change in a whole protein could require a substantial energy input.

The body of work which has been published therefore implicates PME-1 as an inhibitor of PP2A, while PTPA is an activator. Both target the core enzyme – PME-1 maintains it in an intracellular pool, while PTPA activates it. Once activated, the dimer can be rapidly methylated by LCMT-1 ensuring that it is built into a holoenzyme.

Another aspect of enzyme regulation which should always be kept in mind, is the control of the absolute levels of the enzyme within the cells.

Regulating the total amount of PP2A in the cell

Knock-out of PP2A-C proves lethal on embryonic level (Gotz *et al.*, 1998), indicating the significance of PP2A in cellular processes. Attempts to investigate PP2A-C by overexpressing it has mostly failed, since there isn't a stable model of PP2A-C overexpression. The reason for this seems to be that there is a strict autoregulatory mechanism at work maintaining PP2A-C levels within certain limits (Baharians *et al.*, 1998). These authors found that this autoregulatory feedback mechanism targets the translational phase of enzyme expression, while protein turnover seems to be less important. This is yet another fascinating phenomenon which emphasizes the critical importance of PP2A-C.

Despite this conclusion by Baharians *et al.* (1998), protein degradation seems to play a role under certain conditions and especially with regards to the other components of the holoenzyme. Strack and colleagues (2004) reported that knock-down of PP2A-A initiated cell death and was associated with a loss of PP2A activity, as well as a proteosomal mediated reduction in the protein levels of PP2A-C, B and B'. Interestingly B'' and B''' (striatin) levels remained stable. The implication of their results is that while the dimeric core enzyme, or trimeric holoenzyme is stable, any components which are not assembled into either of the two are unstable and consequently degraded – a phenomenon which was also reported, or at least implicated, by others (Van Kanegan *et al.*, 2005; Lee *et al.*, 2007). If this is indeed the case, one could easily argue that the expression of PP2A-C is the central most important factor in determining core- and holoenzyme levels, since it has been reported to have a longer half-life than PP2A-A (16.5 hours vs 10 hours – Baharians *et al.*, 1998; and Zhou *et al.*, 2003).

Alpha4 – targeting PP2A-C for degradation?

Adding to the number of potential molecules that can bind to PP2A, Murata *et al.* reported in 1997 that a protein called α4 has the ability to directly bind to PP2A-C. Later work confirmed that the ubiquitously expressed α4 binds directly to the catalytic subunit, competing with PP2A-A, PME-1, LCMT-1 and okadaic acid (Kloeker *et* al., 2003; Yang *et al.*, 2007; Kong *et al.*, 2009; Migueleti *et al.*, 2012). The implication of this is that PP2A-C can exist in the cell as a dimer with α4, which in the process also reduces PP2A activity (Nanahoshi *et al*.; 1999).

Despite α4 inhibiting PP2A-C, Kong and colleagues (2009) found that α4 is of critical importance for protein phosphatase stability, since deleting it was associated with a progressive reduction in PP2A activity in conjunction with a loss in PP2A-A. It is also noteworthy that α4 also associates with PP4 and PP6 following similar dynamics as with PP2A-C. This however leads to the question: What is the function of α4?

There are three different possibilities, which are probably all true to differing degrees:

1.) α4 has the ability to bind both PP2A-C and another protein, Midline 1 (MID1), simultaneously. MID1 is an E3 ubiquitin ligase – in other words it is involved in the final step of ubiquitination of a protein by acting as a type of scaffold bringing an activated ubiquitin group into the proximity of the substrate (Calise & Powell, 2013), thereby "tagging" the substrate protein for proteosomal degradation. The obvious conclusion is therefore that α4 is involved in the MID1-mediated degradation of PP2A-C. In fact this is one of the proposed mechanisms of the congenital disorder Opitz syndrome, which is molecularly characterized by an increase in the cytosolic levels of PP2A (Trockenbacher *et al.*, 2001). MID1 has also been shown to simply inhibit PP2A (Collison *et al*, 2012). A geometrically opposing function for α4 which involve MID1 has however also been proposed.
2.) McConnel and colleagues (2010) found that although PP2A-C, bound to the α4-MID1 complex was ubiquitinated, this was not associated with the degradation of PP2A-C; rather it seemed to protect PP2A-C. They suggested a mechanism whereby PP2A-C is only mono-ubiquitinated, followed by the binding of an "ubiquitin-interacting motif" in α4 to the ubiquitin group, thereby "capping" it and preventing the addition of more ubiquitin groups. This prevention of the poly-ubiquitination of PP2A-C might explain how α4 protects PP2A-C against degradation (Kong *et al.*, 2009).
3.) Two studies have found that α4 is also involved in targeting PP2A-C to specific locations (Fielhaber *et* al., 2009) and substrates (Prickett *et* al., 2007) within the cell. This identifies α4 as a scaffold protein which simply brings PP2A into the proximity of its intended substrates.

Taken together the scaffold function of α4 best describes its role in living cells. The eventual effect of α4 association with PP2A-C is therefore dependent on the eventual cellular / substrate target associated with α4 scaffolding in that specific context. α4 can therefore possibly even be considered as an alternative scaffold to PP2A-A. It is however intriguing that α4 association has been implicated with the inhibition of PP2A-C, reportedly via an allosteric mechanism (Nanahoshi *et al*.; 1999; Smetana *et al.*, 2007). α4 is however not the only protein that can bind to, and in the process inhibit, PP2A-C.

Other proteins which can bind to PP2A-C

As mentioned in the beginning of this mini-review, the key to PP2A's wide range of substrates and cellular processes it mediates, lies in its modularity; the fact that it consists of units which can be assembled in different combinations to generate a wide array of substrate specificities, cellular locations and enzyme kinetics. Besides the proteins that have already been mentioned, the following molecules can also bind to and – in each case inhibit – the activity of PP2A.

- In 1995 Li and colleagues isolated two potent endogenous inhibitors of PP2A from bovine kidney: I_1^{PP2A} and I_2^{PP2A}. These inhibitors were shown to be highly specific for PP2A, but

interestingly only inhibited the phosphatase activity of PP2A directed at specific substrates and failed to inhibit PP2A's autodephosphorylation. A year later the same investigators determined the identities of these two inhibitors: I_1^{PP2A} is also known as "putative histocompatibility leukocyte antigens class II-associated protein I" (PHAP-I), while I_2^{PP2A} proved to be a truncated form of SET (Suvar3-9, enhancer of zeste, trithorax). PHAP-I has been shown to be part of a family of PP2A inhibitors known as the acidic nuclear phosphoprotein 32 (ANP32) family (Santa-Coloma, 2003). These proteins are also targets of modulation and in 2012 Irie *et al*. reported that the PP2A inhibitory function of SET could be reduced by its phosphorylation. On the other hand, phosphorylation of ANP32e is a prerequisite for it to interact with PP2A (Costanzo *et al.*, 2006).

- In yeast Tip41-like proteins have been found to exert some of their function by regulating PP2A (Fenyvuesvolgyi *et al.*, 2005). The mammalian ortholog of Tip41 is TOR signalling pathway regulator-like (TIPRL), also known as TIP41. TIPRL has been shown to also bind PP2A-C at a site distant from the binding site of α4, a trimer can therefore be formed between PP2A-C, α4 and TIPRL. As is the case for α4, TIPRL exerts an allosteric inhibitory effect on PP2A-C (Smetana & Zanchin, 2007).
- Simian virus 40 (SV40) small-t antigen (small-t) has the ability to bind to either PP2A-A or the core enzyme, in the process competing with the regulator subunit for a position in the holoenzyme and inhibiting the activity of PP2A in a substrate-specific manner (Yang *et al.*, 1991). PP2A is therefore one of the targets for simian virus 40 and polyoma virus mediated cellular transformation (Janssens & Goris, 2001). Small-t presents as a useful experimental tool for the modulation of PP2A (see for example Andrabi *et al.*, 2007).

Conclusion

The regulation of PP2A activity is a vast and complex process, involving numerous regulator proteins. For a review concerning the dynamics of PP2A assembly, see Janssens *et al*. (2008). The reader is also referred to Sents and colleagues (2012) who suggested a model incorporating these various role-players in an attempt to generate a cohesive understanding of PP2A. Their model is built on the premise that a free active catalytic subunit would be catastrophic in a cell and therefore needs to be tightly controlled, as well as protected from degradation. A summary of their model, incorporating the information discussed here combined with some conjecture, is as follows (see Figure 3.1.):

Newly synthesized PP2A-C is stabilized in an inactive state in complex with α4, and possibly also TIPRL – thereby forming a storage depot of PP2A-C. α4 is centrally placed to regulate the rate of PP2A-C MID1 mediated proteosomal degradation, as well as intracellular translocation of PP2A-C *alone*.

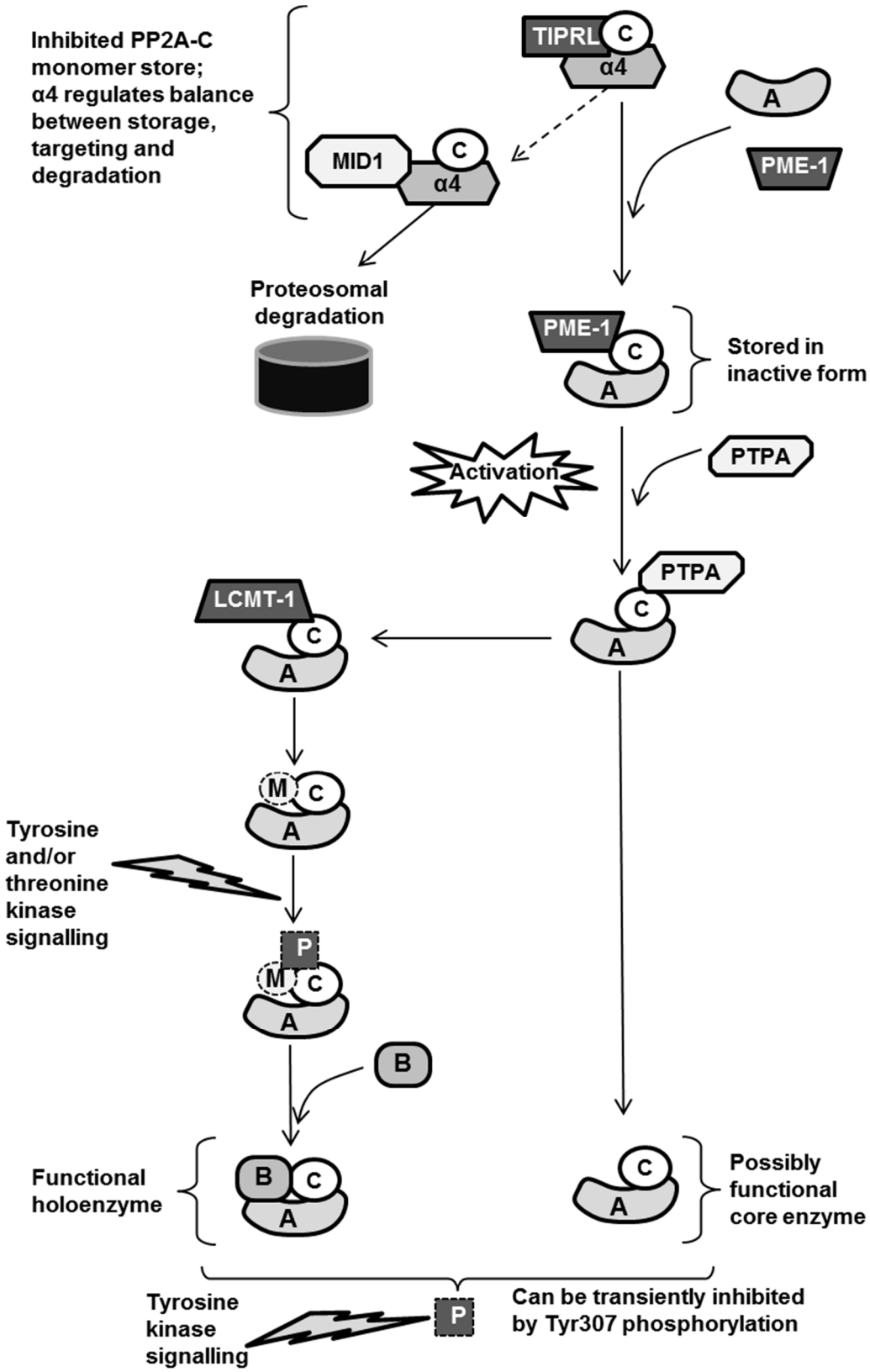


Figure 3.1. Diagrammatic presentation of the regulation of PP2A. Storage depots of PP2A-C monomer or core enzyme must be inactivated and stabilized by α4, TIPRL or PME-1. Following activation by PTPA, the core enzyme can be methylated by LCTM-1, or phosphorylated at tyr307 or thr304 – thereby regulating B subunit recruitment. After PTPA activation, the enzyme can also be transiently inactivated through tyr307 phosphorylation.

Activating PP2A-C for its cellular functions is a multistep process. First PP2A-A must replace α4 (and probably also TIPRL). This resulting core enzyme could possibly participate in cellular processes (after activation by PTPA), or (more likely) also go into a storage depot from which core enzyme can rapidly be withdrawn. This dimeric store is maintained and stabilized in an inactive form by PME-1, which will also ensure that the dimer remains unmethylated.

If the core enzyme needs to be recruited for cellular functions, it will have to be activated by PTPA which catalyses the necessary conformational change. As soon as the core enzyme is active it will become methylated by LCMT-1, in the process facilitating the addition of a regulatory subunit to the core enzyme. The result is the release of a holoenzyme with a specific activity, intracellular location and substrate specificity.

Phosphorylation also influences regulatory subunit selection, so the phosphorylation of thr304 and tyr307 might present a way in which intracellular signalling intercepts and contributes to the determination of the holoenzyme assembly. The phosphorylation of tyr307 also presents an opportunity for tyrosine kinase mediated regulation of PP2A activity. Due to its autodephosphorylation, this form of inhibition is transient and allows for the short-term phosphorylation of substrate proteins in conjunction with tyrosine kinase mediated signalling. Phosphorylation of the regulatory subunits is another way in which PP2A activity can be modulated by signalling pathways.

This is a very simple model which ignores some of the dynamics of PP2A function and regulation. Questions remain, such as: What is the physiological importance of core enzyme activity? What determines the selection of the regulatory subunit? How can such a wide variety of subunits be recruited by a single “on/off” switch (methylated / nonmethylated)? Some have found that the nonmethylated form is ideal for inactive storage, while others have found that methylation status has no effect on enzyme activity, so what is the link then between methylation and function? What determines the selection of binding to α4 versus PP2A-A? There is therefore still a lot of research depth left with regards to PP2A.

Protein phosphatase 2A in the heart

As discussed in the previous sections, PP2A has emerged as an incredibly complex enzyme. The body of work which has already been done in an attempt to elucidate its mysteries has however laid the foundation to better investigate and understand its role and contribution in general pathologies. One such pathology is ischaemic heart disease. Although the original work done in the 1990’s concerning the effects of phosphatase inhibition (Xiuhua *et al.*, 1997; Armstrong *et al.*, 1997; Armstrong *et al.*, 1998; Weinbrenner *et al.* 1998; Barancik *et al.*, 1999; Isotani *et al.* 2002) showed the importance of the phosphatases in this setting, researchers simply didn’t have enough

information to contextualize and expand on their results. However, in light of all the new knowledge we have to our disposal, it is an opportune time to re-examine PP2A in the heart. One such study is the recently published description of PP2A in the heart by DeGrande and colleagues (2013).

Applying Western Blotting these authors found that 9 regulatory subunits are expressed in human heart tissue: B (PR55 α, β); B'' (PR72); B''' (PR93/PR110); B' (PR56 α, β, γ, δ, ε), implying that there are numerous PP2A holoenzymes at work in heart tissue. As expected, in mouse myocytes the regulatory subunits showed differential intracellular distribution, while PP2A-A and –C were spread throughout the cells. Interestingly, they also found that there is a higher expression of PP2A-A and PP2A-C in the right ventricle than in the left ventricle – confirming earlier observations (Lüss *et al.*, 2000). Comparison of regulatory subunit expression between animal species revealed a worrying difference in expression between rodents (mice and rats) and humans – these differences must obviously be kept in mind when interpreting experimental results. It is also noteworthy that they found that PP2A subunit expression changed in pathological models, but in a unique way for each pathology investigated (ischaemic and non-ischaemic heart failure). These changes in expression were more visible on protein level than transcriptional level, indicating the importance of regulation on other levels – such as translation and degradation. They also reported that heart failure *per se* was also associated with an increase in tyr307 phosphorylation and a reduction in methylation (with a concurrent reduction in LCMT-1 expression). This study therefore clearly confirms the importance and participation of PP2A in cardiac pathology.

Motivation, hypothesis and aims

We therefore know that PP2A is present in the heart; it is involved in several processes such as Ca^{2+} homeostasis, contraction, apoptosis and signalling; inhibition of PP2A has been shown to be cardioprotective and its expression is modulated in ischaemic and non-ischaemic heart failure. It should therefore be involved in the acute setting of myocardial I/R injury. Remarkably little is however known concerning the basic dynamics of PP2A participation in this context.

This study aims to address this shortcoming by simply describing PP2A levels and posttranslational modification in unfractionated tissue, as well as in different cellular fractions, as ischaemia progresses and during the clinically relevant first moments of reperfusion.

Due to the explorative nature of this study, it is difficult to predict the outcomes – our hypothesis is therefore very limited. In light of earlier work done in renal tissue (Kobryn and Mandel, 1994), we expect that PP2A will continue to function as an active phosphatase during ischaemia. The implication of this is that the phosphorylation level of PP2A should remain unchanged. As ischaemia progresses, with concomitant reduction in ATP levels, the phosphorylation of PP2A will also decrease. Even in the absence of a cardioprotective intervention, we expect the heart to

respond to I/R stress by attempting to activate its inherent cardioprotective capability. This is dependent on phosphorylation driven survival signalling. Therefore, reperfusion and the generation of ATP should be associated with a transient increase in PP2A phosphorylation.

Ischaemia is associated with a relatively rapid cessation of contraction, while heart tissue still remains viable. PP2A might contribute to this by reducing the phosphorylation and activity of the sarcolemmal L-type Ca^{2+} channel, ($Ca_v1.2$). However, it is also conceivable that PP2A could contribute to Ca^{2+} overload and contracture later in ischaemia and during early reperfusion by modulating PLB, RyR2, as well as TnI. We therefore expect to find a translocation of PP2A between cellular fractions; possibly towards a membrane fraction. This translocation will be associated with changes in the level of methylation of PP2A. Depending on the degree of damage incurred, reperfusion should be associated with the redistribution of PP2A back to its pre-ischaemic intracellular positions, although the time-frame for this is unknown.

Material and Methods

Rationale

To investigate the response of PP2A to ischaemia and reperfusion we focussed our attention on the levels of PP2A-A and PP2A-C at different timepoints in the latter half of an ischaemic incident, as well as during the first moments of reperfusion. We specifically chose to investigate the latter half of ischaemia, since we expected to see the most pronounced effects after a relatively extended period of ischaemia. Our interest in early reperfusion was due to the fact that this has been shown to be a critical period in the genesis of I/R injury and therefore also a window of opportunity for clinical intervention. Since the activity and cellular location of PP2A are influenced by phosphorylation and methylation, these parameters were also measured. All protein and posttranslational modification (PTM) determinations were done using standard Western Blotting techniques (previously described in Chapter 2).

In order to confirm our results we chose to use two different experimental models, namely cell culture and the isolated rat heart. Each of these models were exposed to different periods of ischaemia and reperfusion, as relevant for that experimental model, whereafter samples were collected and analysed using Western Blotting.

Cell culture experiments

Protocols

For the cell culture experiments we utilized a myoblast cell line of cardiac origin: H9c2 cells. Ischaemia was simulated in these cells by exposing them to a combination of hypoxia (0.5% O_2,

5% CO_2 and the balance N_2) and chemically simulated ischaemia (SI) in the form of a modified Esumi buffer (Esumi *et al.*, 1991) containing the metabolic mitochondrial inhibitor sodium dithionate (SDT, 0.5 mM). We first had to determine the optimal maximum duration of SI exposure. For this we assessed cell viability, measured by PI and AV staining, using a Becton Dickinson FACS (fluorescent activated cell sorting) Calibur. This approach allowed us to determine both early and late apoptosis / necrosis. Exposure was considered sufficient if it elicited a significant degree of cell death. Following this appoach we utilized 120 minutes of SI exposure as the maximum ischaemic duration.

See Figure 3.2 for the different protocols of SI and reperfusion which were followed. Briefly, cells were exposed to SI for 60 minutes, 90 minutes or 120 minutes. Following 120 minutes of SI the cells were reperfused. Simulated reperfusion entailed a brief rinse with PBS followed by incubation in normal DMEM growth medium under normoxic conditions for either 15 or 30 minutes.

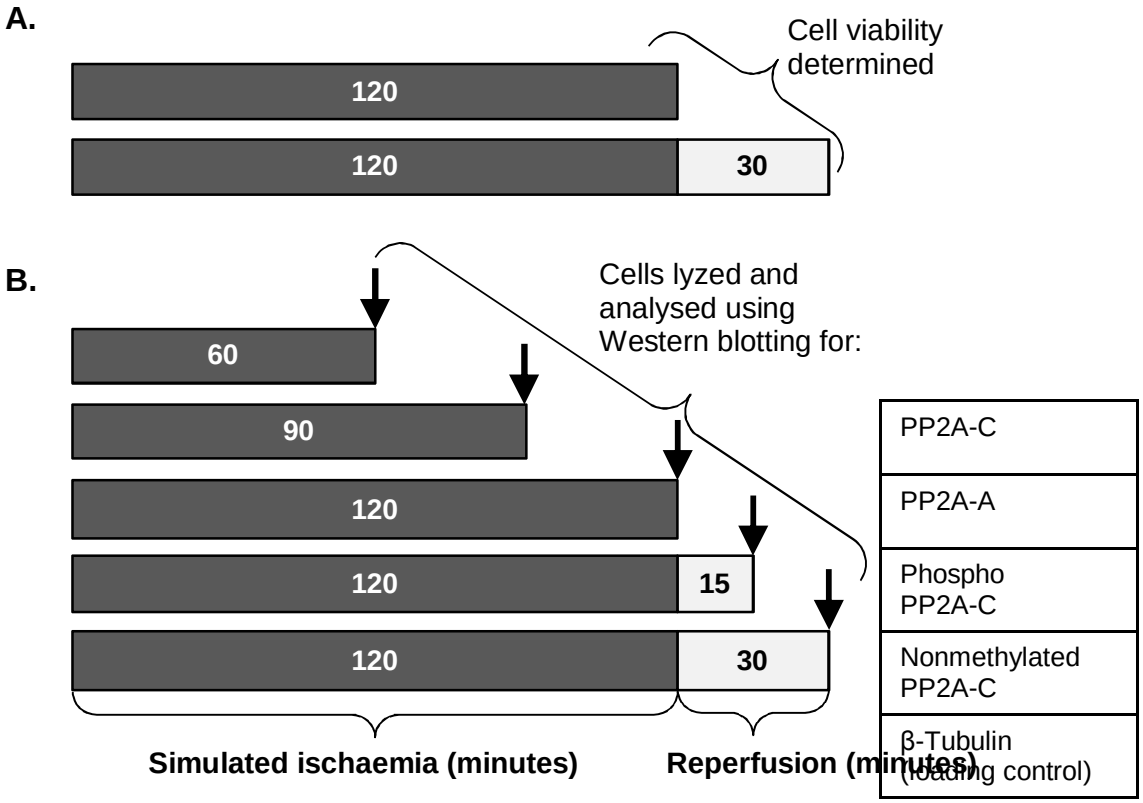


Figure 3.2. Simulated ischaemia and reperfusion in the H9c2 cell model.
(A) To confirm the detrimental effects of our model of simulated ischaemia (SI) and reperfusion, cells were exposed to either 120 minutes SI alone, or 120 minutes SI followed by 30 minutes reperfusion. Following these interventions cell viability was determined using prodium iodide (PI) and Annexin V (AV) staining. (B) After confirming that 120 minutes SI was sufficient to elicit cell damage and death, protein levels and posttranslational modification of PP2A was determined at 60, 90 and 120 minutes SI, as well as 15 and 30 minutes reperfusion.

Western blotting was used to determine the profile of PP2A-C and –A expression, as well as phosphorylation and methylation of PP2A-C, following these different durations of SI or SI/R. Each intervention was paired with a relevant control group. The control groups were exposed to the

same handling as the experimental groups, i.e. PBS washes and growth medium replacement. The only difference was that they received normal DMEM growth medium throughout.

Statistics

Each experimental intervention was repeated three times, on different days. For each repetition of the relevant experimental condition at least three separate culture dishes served as controls, while four to six culture dishes were exposed to SI or reperfusion. Flow cytometry data was originally collected as the percentage of the cells counted presenting with either AV or PI positive staining, or both. As described in Chapter 2, densitometry was applied to quantify the final Western blotting films, thereby expressing the Western blots in terms of pixels. For both end-points measured comparison of data generated on different occasions were facilitated by expressing all the data relative to the mean of the relevant control group and expressed in arbitrary units (AU). Since the control condition remained the same between experimental repetitions, we pooled all the normalized data into a single statistical comparison for each end-point measured.

Since, for each intervention, only one experimental group was compared with its relevant control, data analysis was done using an unpaired T-Test. Depending on the distribution of the data, determined by a D'Agostino & Pearson omnibus normality test, either an unpaired T-test (for Gaussian distributed data), or a Mann-Whitney test (a nonparametric test) was used. A P-value of less than 0.05 was considered significant.

Isolated rat heart experiments

Protocols

As previously described (Chapter 2), hearts were excised from male Wistar rats and quickly mounted on a work heart perfusion apparatus. As was the case for the cell-based experiments we first had to determine which duration of global ischaemia (GI) would be sufficient to elicit significant damage, as determined by the functional recovery of the hearts. Functional recovery is defined as functional performance, as determined by aortic output, cardiac output and total work measurements, at 30 minutes reperfusion relative to pre-ischaemic values. We found that a period of 20 minutes GI was adequate. Therefore, following 40 minutes of stabilisation hearts were exposed to 10, 15 or 20 minutes GI, or 20 minutes GI followed by either 5 or 10 minutes of reperfusion (see Figure 3.3). Tissue was collected at each of these timepoints and stored in liquid nitrogen for later analysis.

As described for the cell based experiments, tissue was analysed using Western Blotting for PP2A-C, PP2A-A, phosphorylated PP2A-C and nonmethylated PP2A-C. Beta tubulin was used as a control for equal loading. Since a large part of the regulation of PP2A involves the cellular

translocation of the enzyme, we also utilized the collected tissue to prepare crude nuclear, cytoplasmic and membrane fractions, as described in Chapter 2. Each of these fractions were then also analysed using Western blotting for the same targets as investigated in the unfractionated preparation.

Control hearts were perfused for a similar duration of time as the hearts exposed to global ischaemia.

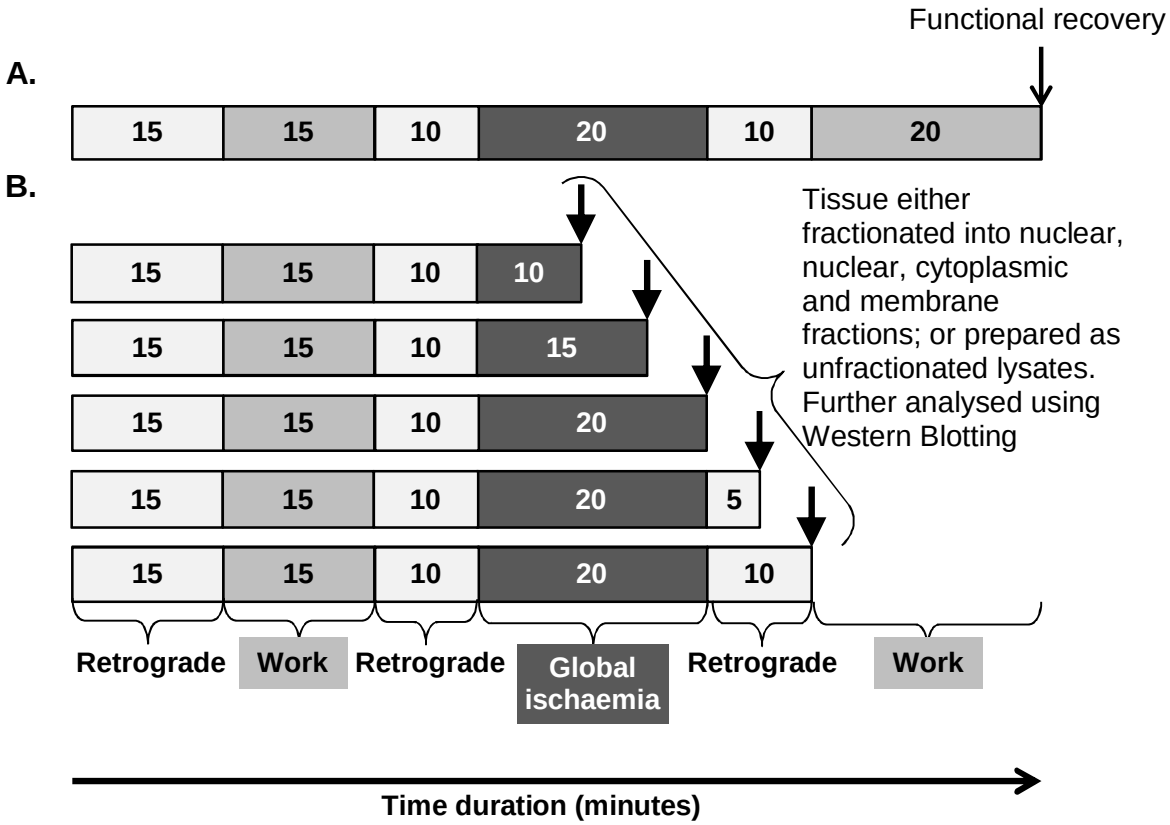


Figure 3.3. Global ischaemia in the isolated rat heart model.
(A.) To determine the effects of global ischaemia (GI) and reperfusion in our model, hearts were stabilised for 40 minutes and then exposed to 20 minutes global ischaemia at a temperature of 36.5°C. This was followed by a total of 30 minutes reperfusion. Functional recovery was then calculated as the post-ischaemic function relative to pre-ischaemic values.
(B.) Having confirmed that 20 minutes GI conferred significant damage, hearts were exposed to 10, 15 or 20 minutes GI; or 20 minutes GI followed by 5 or 10 minutes reperfusion. At each of these timepoints heart tissue was collected and analysed using Western Blotting. For each time point an unfractionated "whole cell" lysate was prepared, as well as fractionated (nuclear, cytoplasmic, membrane) samples.

Statistics

Each timepoint was investigated as a separate experiment with at least three control hearts and four ischaemia/reperfusion hearts. Due to technical limitations, we performed Western Blotting on each timepoint separately. We therefore decided to only directly compare each timepoint with its non-ischaemic control, and not timepoints with each other. Statistical analysis was therefore done using an unpaired T-test, where a P-value of less than 0.05 was considered as significant.

Results

Cell culture experiments

Cell viability following simulated ischaemia and reperfusion

At the onset of this project we did not have an established SI model for the H9c2 cells in our laboratory. We therefore first had to setup a suitable model of SI and reperfusion (data not shown). We wanted a cell based model of ischaemia which could be comparable to our isolated rat heart ischaemic model. We therefore decided to not only expose the cells to hypoxia, but also to some of the other aberrations associated with ischaemia.

To this end we combined hypoxia (0.5% O_2) with a modified Esumi buffer containing elevated potassium concentrations, no glucose, low FBS, low pH (6.4), reduced buffering capacity (HEPES buffer at a concentration of 4 mM versus 20 mM in a normal HEPES buffer) and lactate (as discusssed in Chapter 2). H9c2 cells however have a high resistance to hypoxia and ischaemia, possibly due to, amongst other factors, their low metabolic requirements compared to beating cardiomyocytes. To increase the potency of our SI, we also included 0.5 mM of sodium dithionate (SDT), a reversible metabolic inhibitor. We found that exposure of the H9c2 cells to this SI buffer for a period of 2 hours elicited a slight, yet significant degree of cell death (Figure 3.4), both in terms of early apoptosis (AV staining: Control: 1.00±0.14 arbitrary units (AU) vs 2h SI: 3.44±0.97 AU; $p<0.001$) and late apoptosis (AV and PI positive: Control: 1.00±0.17 AU vs 2h SI: 2.48±0.35 AU; $p<0.001$). There was also a significantly smaller population of viable cells (negative staining for either AV or PI) after 2 hours SI (Control: 1.00±0.01 AU vs 2h SI: 0.83±0.04 AU, $P<0.0001$). Similarly, 2 hours SI followed by 30 minutes reperfusion also elicited significant cell death, of approximately the same magnitude as SI alone, with regards to early and late apoptosis, as well as a reduction in the number of viable cells (Figure 3.5).

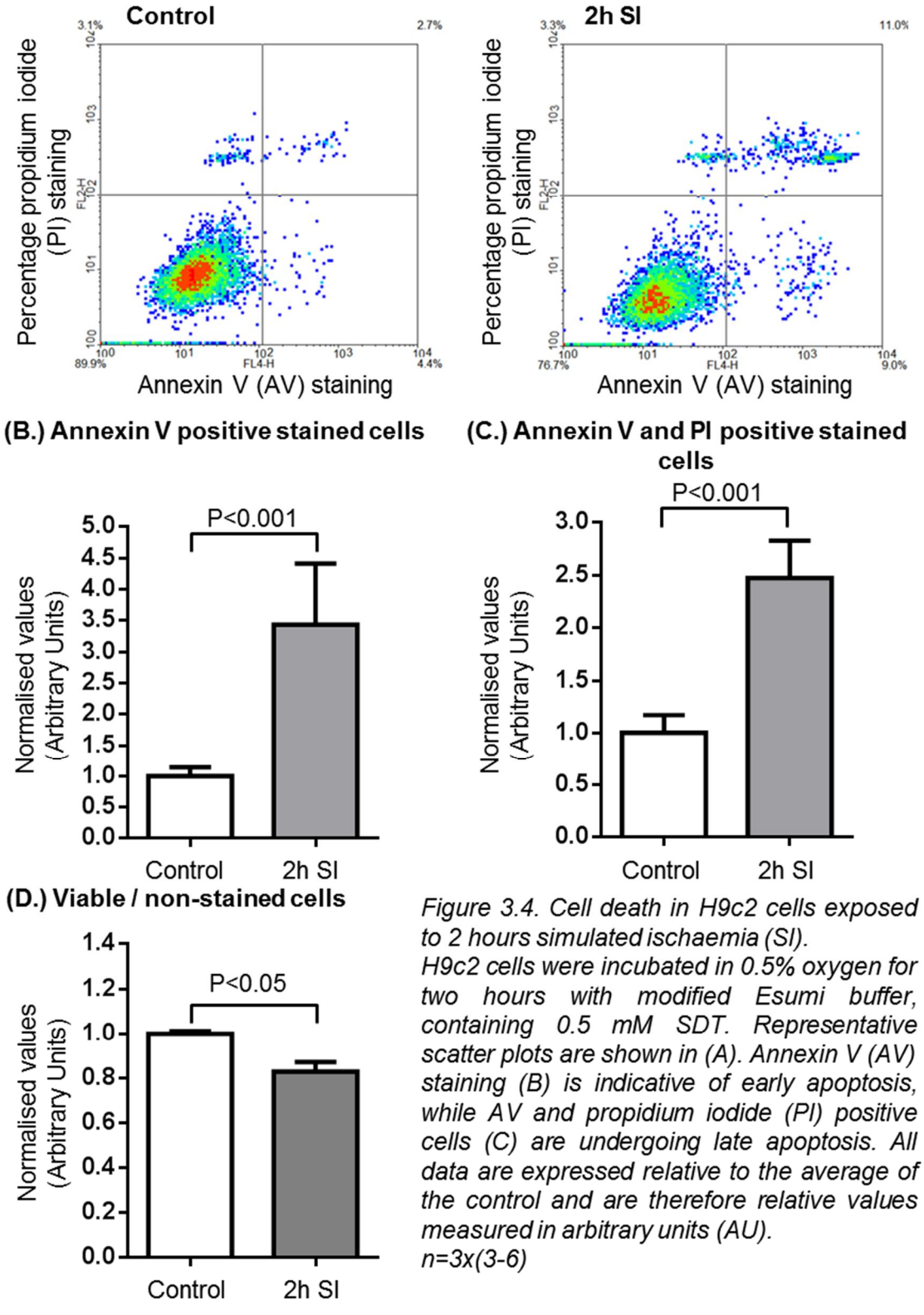


Figure 3.4. Cell death in H9c2 cells exposed to 2 hours simulated ischaemia (SI).
H9c2 cells were incubated in 0.5% oxygen for two hours with modified Esumi buffer, containing 0.5 mM SDT. Representative scatter plots are shown in (A). Annexin V (AV) staining (B) is indicative of early apoptosis, while AV and propidium iodide (PI) positive cells (C) are undergoing late apoptosis. All data are expressed relative to the average of the control and are therefore relative values measured in arbitrary units (AU).
n=3x(3-6)

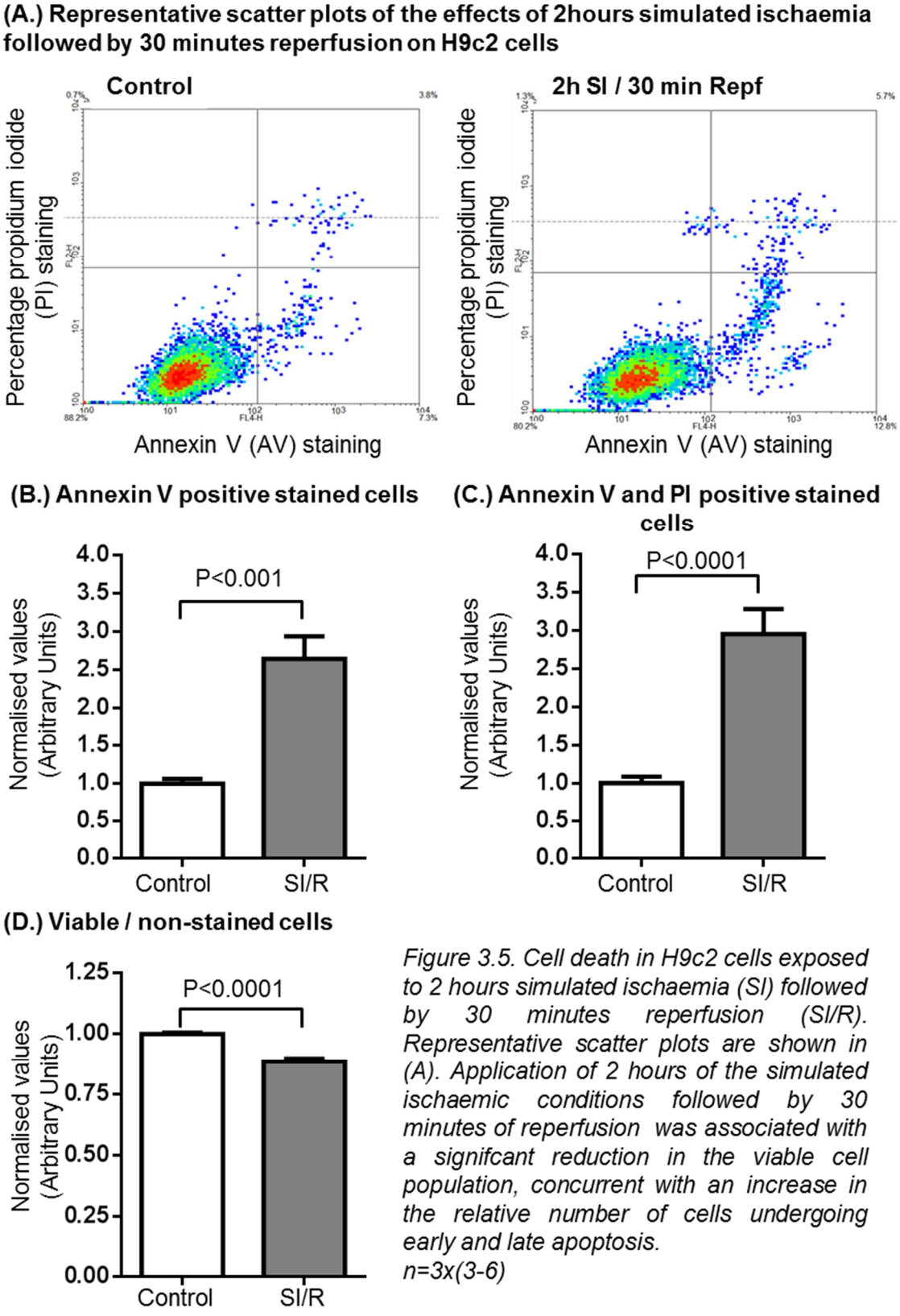


Figure 3.5. Cell death in H9c2 cells exposed to 2 hours simulated ischaemia (SI) followed by 30 minutes reperfusion (SI/R). Representative scatter plots are shown in (A). Application of 2 hours of the simulated ischaemic conditions followed by 30 minutes of reperfusion was associated with a signifcant reduction in the viable cell population, concurrent with an increase in the relative number of cells undergoing early and late apoptosis.
n=3x(3-6)

PP2A during late ischaemia and early reperfusion

Having established an SI/reperfusion (SIR) intervention which was associated with a significant degree of cell injury and death in the H9c2 cells, we proceeded with the characterization of PP2A during the latter half of simulated ischaemia and early reperfusion. We focussed on the levels of total PP2A-C and PP2A-A, as well as tyr307 phosphorylation and leu309 methylation of PP2A-C. Beta-tubulin was also blotted for as a loading control.

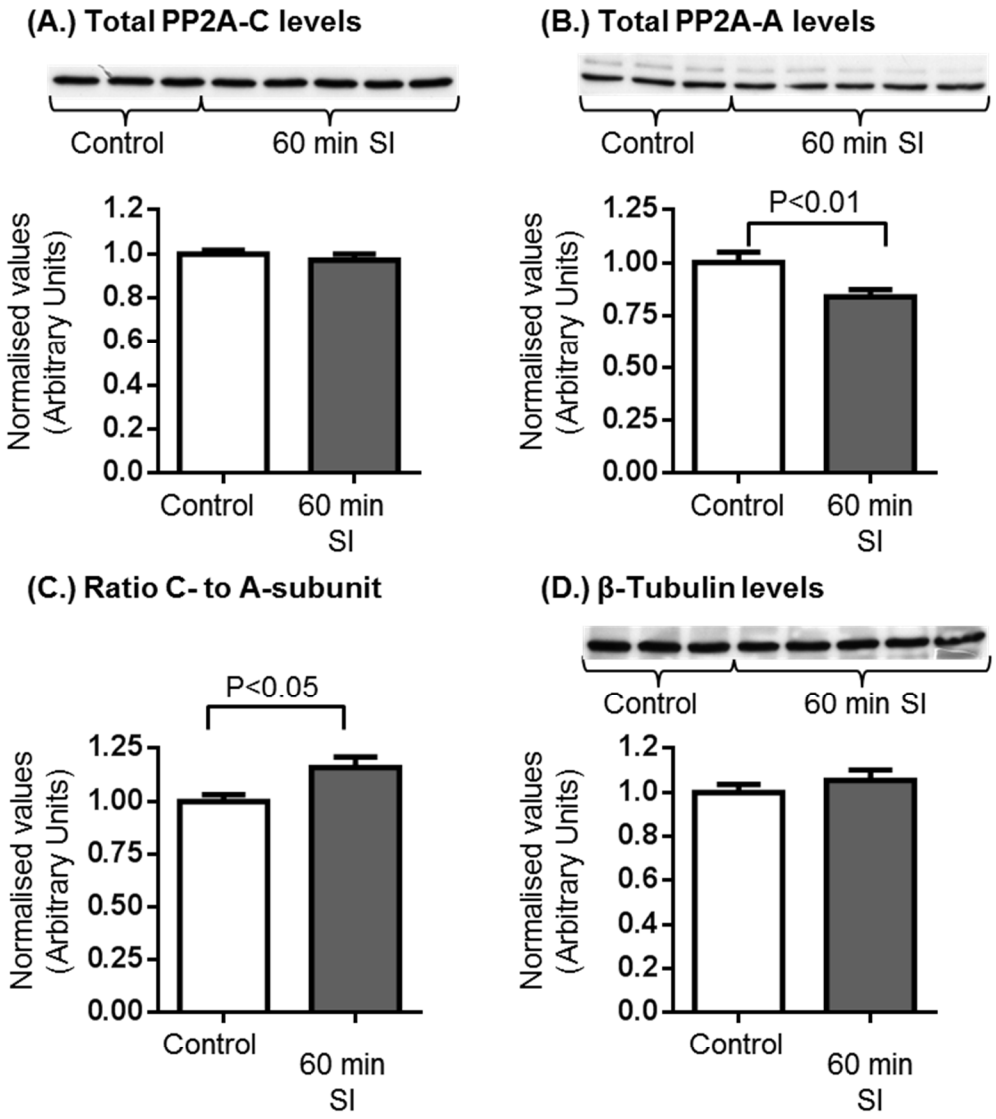


Figure 3.6. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) following 60 minutes of simulated ischaemia. We also calculated the ratio between PP2A-C and PP2A-A to determine if 60 min SI led to a change in the amounts of these subunits relative to each other. Simulated ischaemia reduced the amount of PP2A-A, with an accompanying increase in the ratio of PP2A-C to –A.
n=3x(3-5); of the three repetitions done, one representative blot of the results is shown.

60, 90 and 120 minutes of simulated ischaemia

Following 60 minutes of exposure to simulated ischaemic conditions (Figure 3.6) there was a significant reduction in the levels of PP2A-A (Control: 1.00±0.05 AU vs 60 min SI: 0.84±0.03 AU; $P<0.01$) in the absence of any changes in PP2A-C levels. This reduction was also reflected by a shift in the ratio of PP2A-C to PP2A-A (Control: 1.00±0.03 AU vs 60 min SI: 1.16±0.05 AU; $P<0.05$), indicating that there was now more PP2A-C than PP2A-A relative to control conditions.

These observations regarding the ratio of PP2A-C to – A (PP2A-C/A) can be explained as follows: most of PP2A inside a cell consist of either the dimer or the trimeric holoenzyme. In both these forms PP2A-C and –A is present in a ratio of 1:1. We would therefore expect that under normal conditions there should be approximately an equal number of both PP2A-C and –A molecules within a cell. Western Blotting does not allow for the determination of exact protein concentration in the tissue. We therefore do not have the data to determine the actual ratio of PP2A-C molecules relative to PP2A-A. We do however have an immunoreactive signal representing PP2A-C and PP2A-A and the response in their levels to different situations. To optimise the data we therefore decided to also express these signals relative to each other, similarly as is done for the expression of phosphorylated signal relative to its total, in order to give an indication of changes in the values of PP2A-C and PP2A-A relative to each other – and therefore per implication also give us information regarding dimer and trimer composition. Since we do not have absolute values, we can only express our data relative to a central reference point – which is obviously the control condition. This means that control has the designated value of “1”, not to be confused with an actual 1:1 ratio of absolute values. The PP2A-C/A ratio therefore serves as an indication whether there are changes in dimer or trimer assembly. The increase we see after 60 minutes SI indicates that there is more PP2A-C in the cell than PP2A-A, relative to pre-ischaemic control conditions. This implies that there must be PP2A-C which is not built into either a dimer or a trimer at 60 minutes SI. This might be the case under control conditions as well, since we can’t measure if the absolute ratio is 1:1 under control conditions. However after 60 minutes there is more PP2A-C not associated with PP2A-A than under control conditions.

Along with these changes in PP2A-A relative to PP2A-C, there was an increase in the nonmethylated signal (Figure 3.7) indicating a reduction in methylation of PP2A-C – both in absolute terms (i.e. the amount of PP2A-C nonmethylated – Control: 1.00±0.05 AU vs 60 min SI: 1.17±0.04 AU; $P<0.05$) and relative to total PP2A-C (i.e. the degree of nonmethylation of the PP2A-C population – Control: 0.99±0.06 AU vs 60 min SI: 1.19±0.03; $P<0.01$).

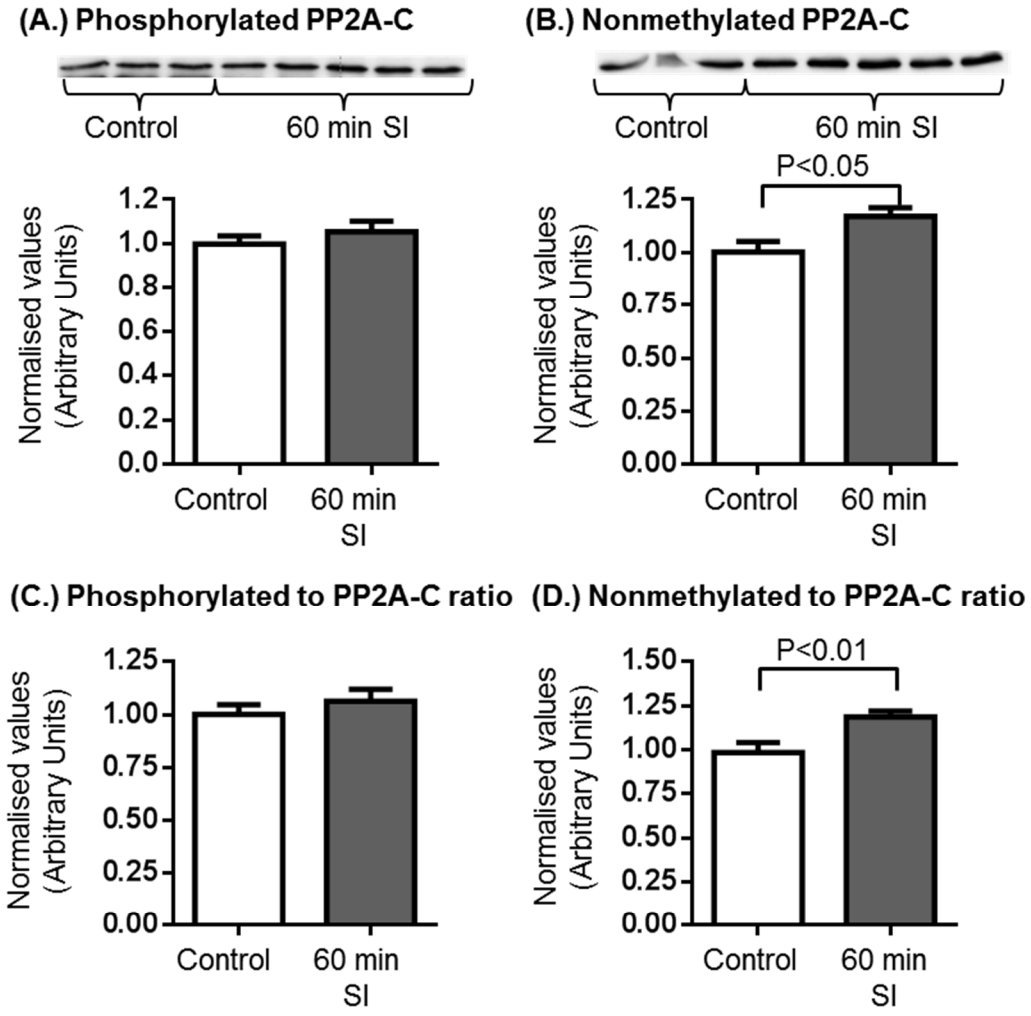


Figure 3.7. Phosphorylation (A & C) and methylation (B & D) of PP2A-C following 60 minutes of simulated ischaemia. Sixty minutes of SI was associated with an increase in nonmethylated PP2A-C, both in absolute terms, as well as relative to total PP2A-C.
n=3x(3-5); one representative blot of the results is shown.

Surprisingly, no differences were observed with regards to total protein levels (Figure 3.8) or phosphorylation and methylation (Figure 3.9) at 90 minutes of SI. This implies that the differences observed after 60 minutes were only transient and probably part of a relatively initial response to SI which includes PP2A participation.

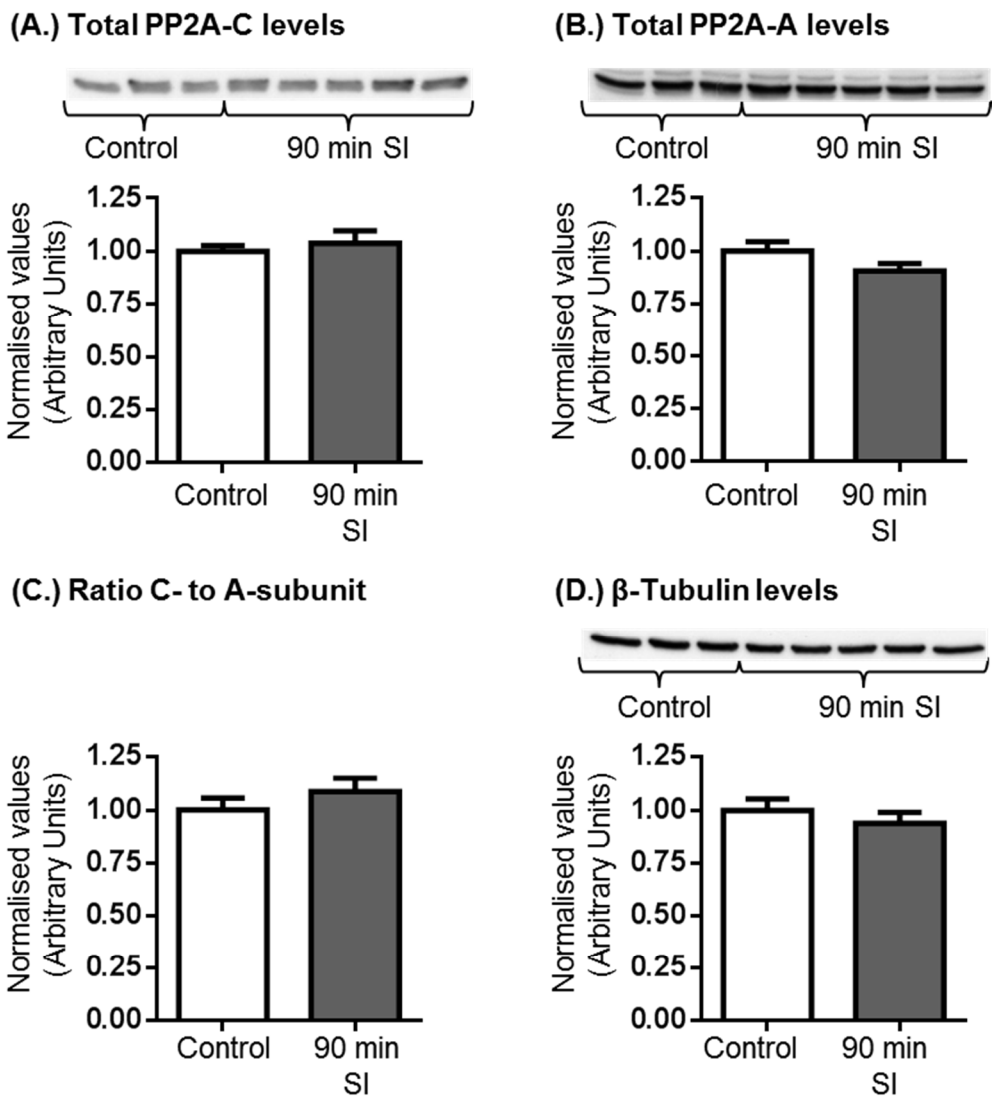


Figure 3.8. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) following 90 minutes of simulated ischaemia. Ninety minutes of SI failed to induce any changes in protein levels.
n=3x(3-5); one representative blot of the results is shown.

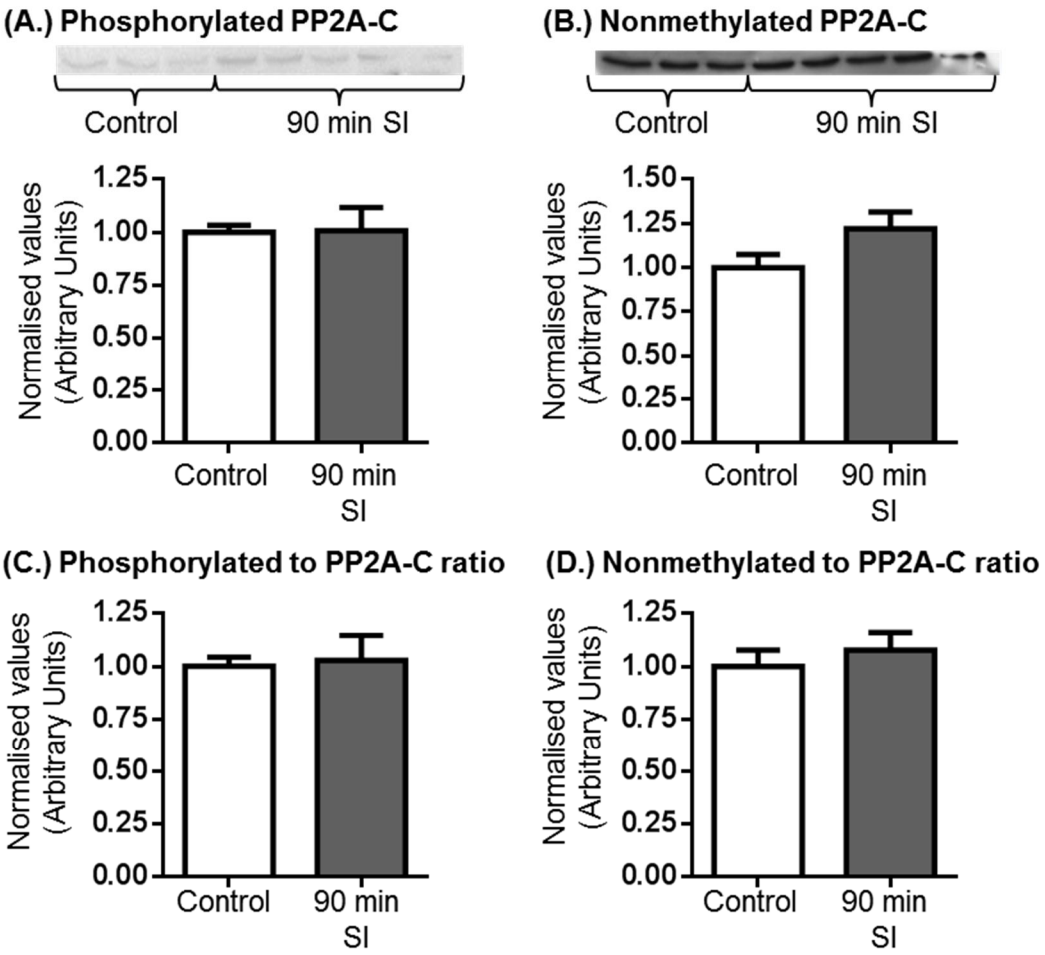


Figure 3.9. Phosphorylation (A & C) and methylation (B & D) of PP2A-C following 90 minutes of simulated ischaemia. Posttranslational modification of PP2A-C was unaffected by 90 minutes of SI.
n=3x(3-5); one representative blot of the results is shown.

Although there were no significant differences relative to control concerning the levels of PP2A-C and –A at 120 minutes of SI (Figure 3.10), there was a significant increase in PP2A-C relative to PP2A-A (Control: 1.00±0.03 AU vs 120 min SI: 1.17±0.04 AU, $P<0.01$). As was the case after 60 minutes, this indicates that there must be PP2A-C in the cell not assembled into the canonical dimer or trimer, relative to control conditions. This shift is evidence of slight, statistically insignificant changes in both PP2A-A and PP2A-C. It therefore seems that the PP2A-C/A value serves to also magnify small changes in PP2A-C and PP2A-A levels, which might be missed otherwise.

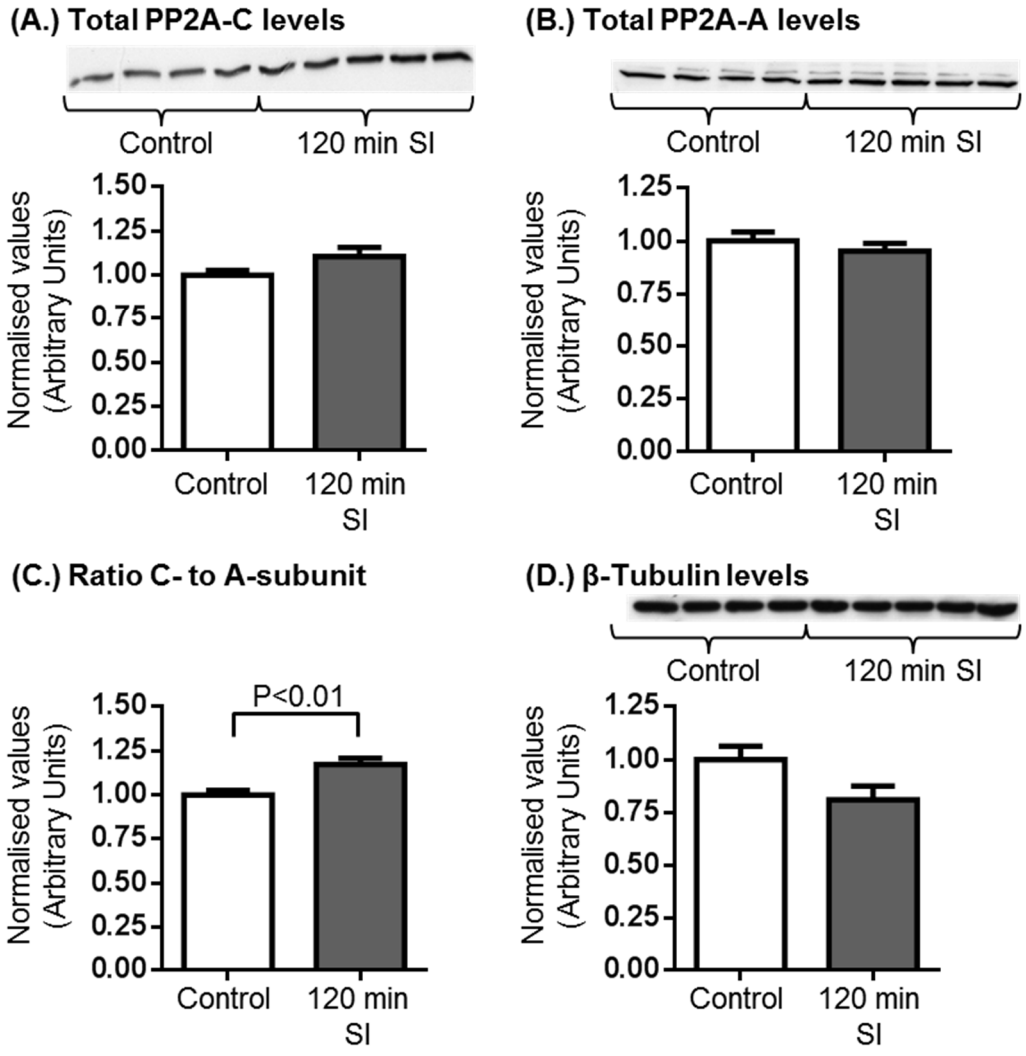


Figure 3.10. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) following 120 minutes of simulated ischaemia. Although there were no significant changes in the levels of either PP2A-A or –C, there was an increase in the levels of PP2A-C relative to PP2A-A.
n=3x(3-5); one representative blot of the results is shown.

Similarly as at 60 minutes, this shift is associated with an increase in absolute nonmethylated PP2A-C levels (Control: 1.00±0.05 AU vs 120 min SI: 1.18±0.06 AU; $P<0.05$). This increase is however not enough to reflect an increase in the degree of nonmethylation of PP2A-C (Figure 3.11).

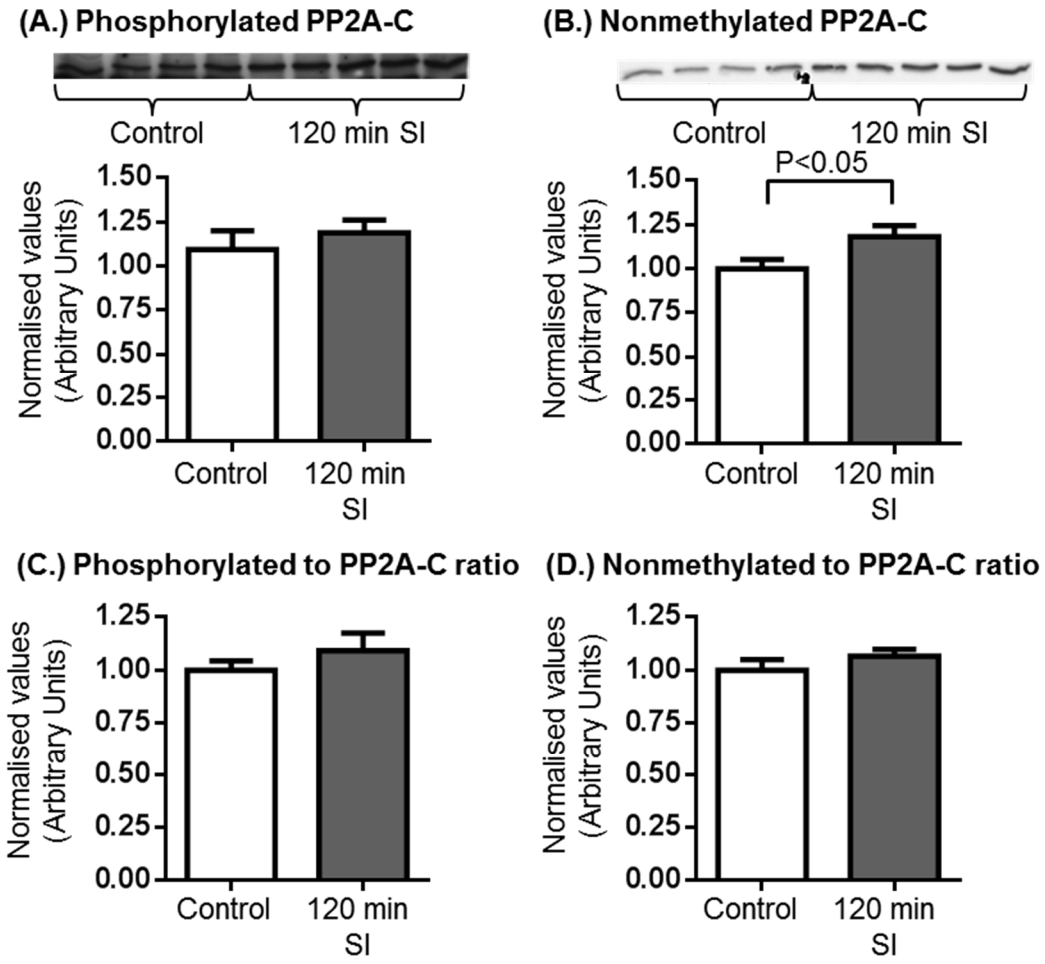


Figure 3.11. Phosphorylation (A & C) and methylation (B & D) of PP2A-C following 120 minutes of simulated ischaemia. After 2 hours of SI there was an increase in nonmethylated PP2A-C, although relative to total PP2A-C this was not signifcant. n=3x(3-5); one representative blot of the results is shown.

15 and 30 minutes of reperfusion

Interestingly, 15 minutes of reperfusion was associated with a reduction in PP2A-A levels (Control: 1.00±0.06 AU vs 5 min Repf: 0.84±0.04; P<0.05). The importance of this is however doubtful, since there is no accompanying shift in the ratio of PP2A-C to –A, implying no changes in trimer or dimer composition (Figure 3.12). There was also no associated change in methylation as observed at 60 and 120 minutes SI (Figure 3.13). There was however a reduction in the amount of phosphorylated PP2A-C (Control: 1.00±0.06 AU vs 15 min Repf: 0.84±0.04 AU; P<0.05). The importance of this is also doubtful, since there was no observed reduction in the degree of phosphorylation of the population of PP2A-C as a whole.

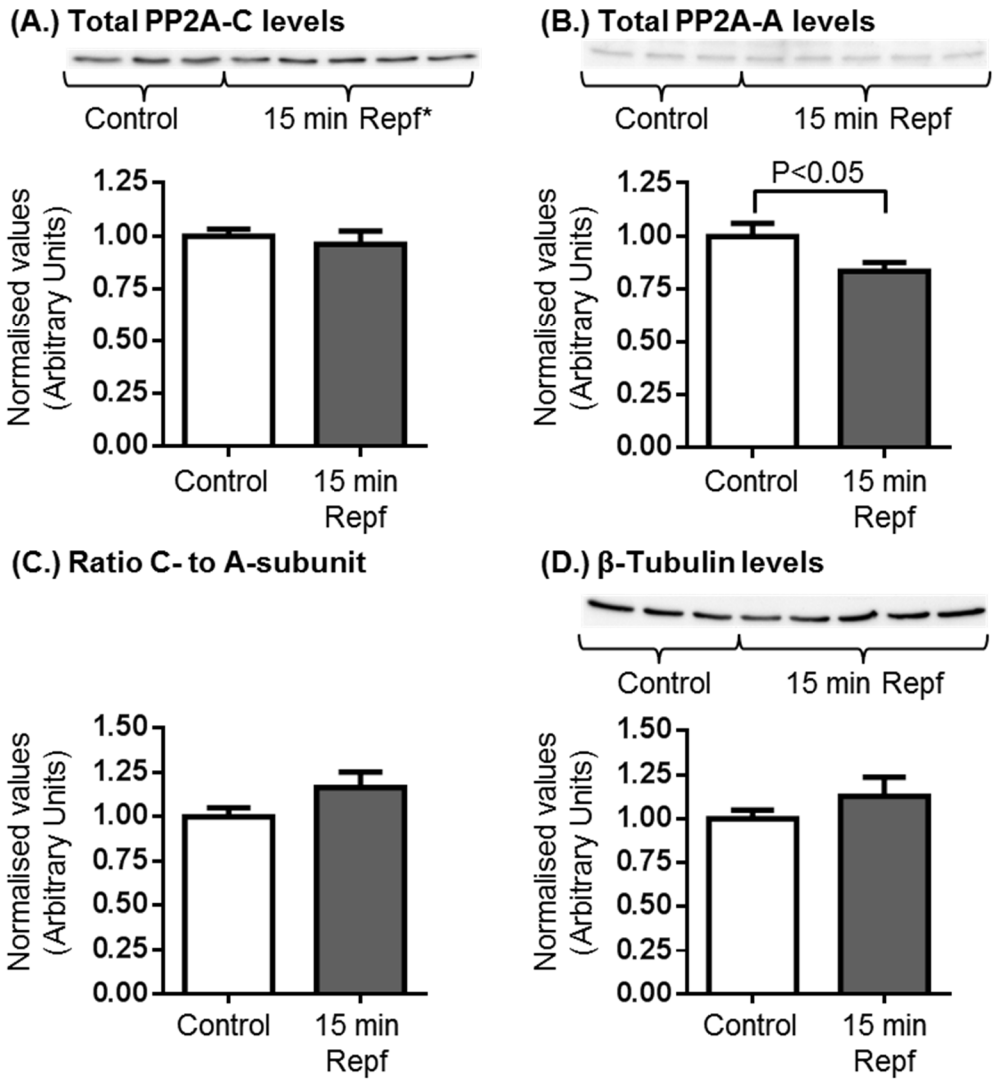


Figure 3.12. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 15 minutes reperfusion following 120 minutes sustained ischaemia. Reperfusion was associated with a reduction in PP2A-A, while PP2A-C remained unchanged. The ratio between PP2A-C and –A also remained statistically unchanged.
n=3x(3-5); one representative blot of the results is shown.
**Repf: Reperfusion*

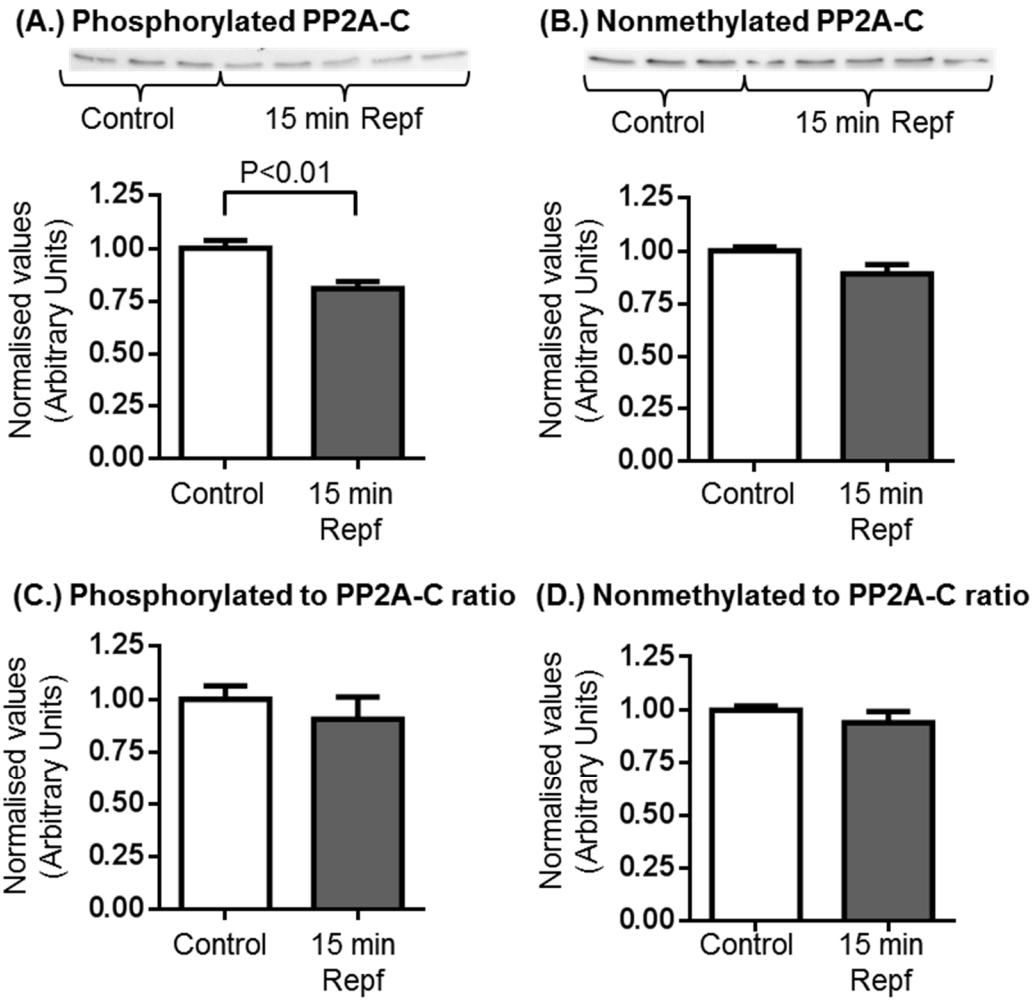


Figure 3.13. Phosphorylation (A & C) and methylation (B & D) of PP2A-C after 15 minutes of reperfusion. The phosphorylation of PP2A-C is reduced, although this is not significant relative to the total levels of PP2A-C.
n=3x(3-5); one representative blot of the results is shown.

It seemed that by 30 minutes of reperfusion the intracellular milieu had returned to normal, as evidenced by the absence of any changes in PP2A levels (Figure 3.14) or posttranslational modification (Figure 3.15).

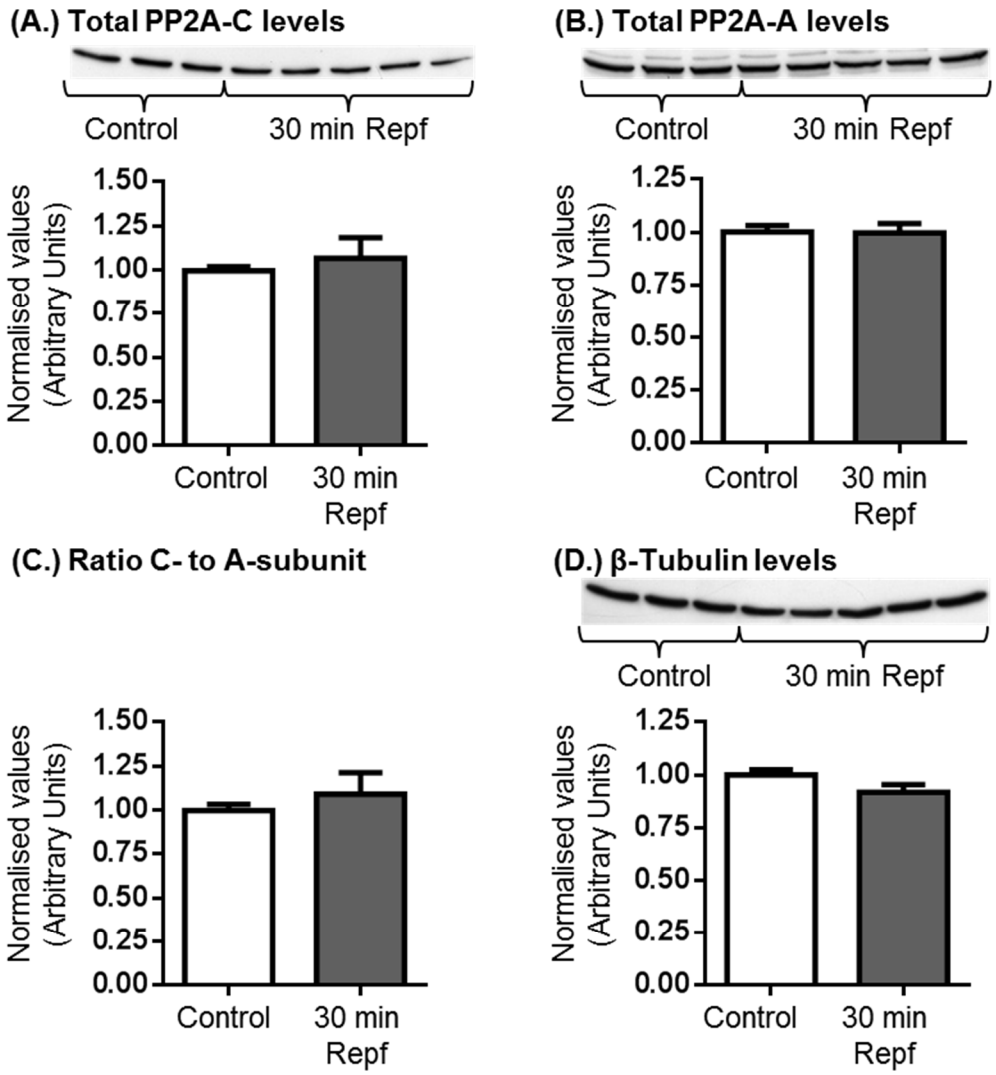


Figure 3.14. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 30 minutes reperfusion following 120 minutes sustained ischaemia. At this stage of reperfusion there were no changes in total PP2A-C or –A. n=3x(3-5); one representative blot of the results is shown.

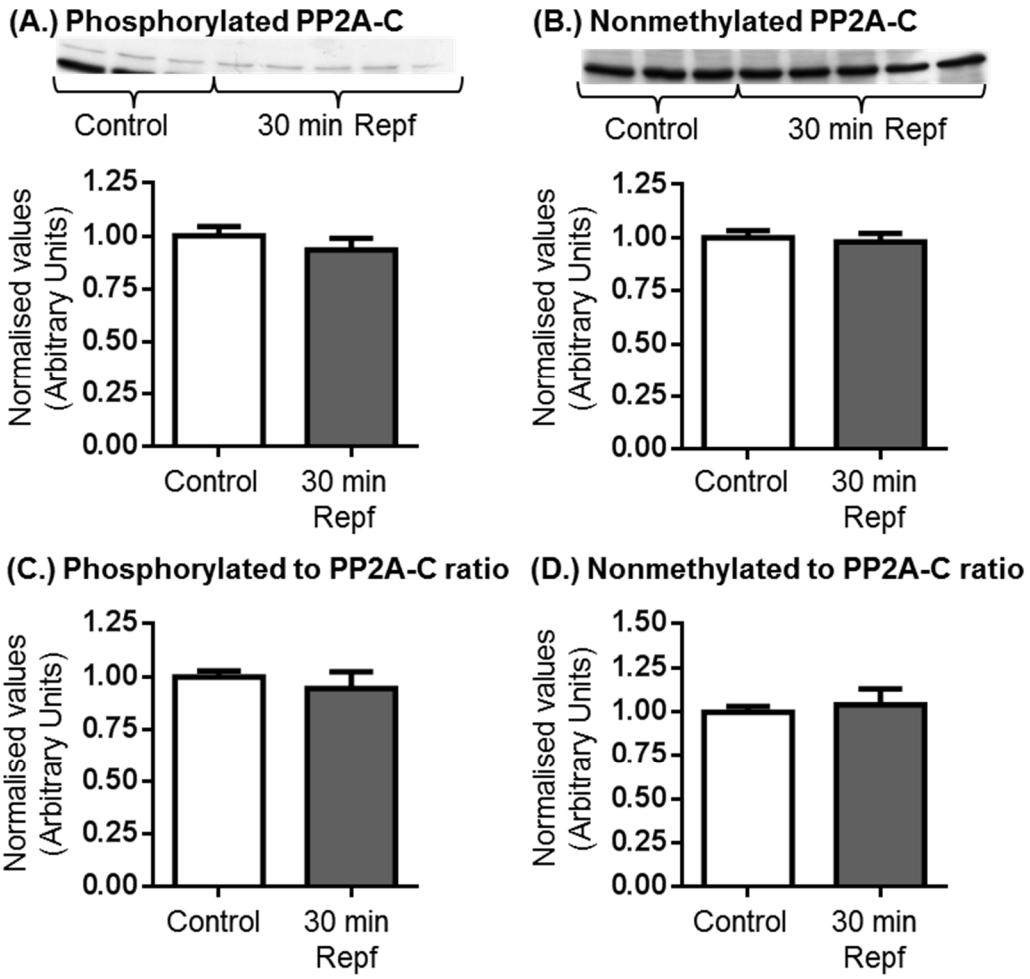


Figure 3.15. Phosphorylation (A & C) and methylation (B & D) of PP2A-C following 30 minutes of reperfusion. The posttranslational modification of PP2A-C was unaffected by 30 minutes reperfusion.
n=3x(3-5); one representative blot of the results is shown.

Isolated rat heart experiments

Functional recovery following an ichaemia / reperfusion intervention

Isolated rat hearts were allowed to stabilize on the perfusion apparatus for a period of 40 minutes followed by 20 minutes of GI during which time the heart received no perfusion with oxygenated Krebs-Henseleit buffer and was kept warm at 36.5 °C. This was followed by 30 minutes of reperfusion, at which time the functional ability of the hearts was assessed and expressed as a percentage of pre-ischaemic function. This combination of GI and reperfusion was associated with a significant reduction in functional performance of the hearts (Table 3.2.), indicating that 20 minutes of ischaemia already exerted a profoundly detrimental effect and could therefore be used to assess PP2A in I/R injury.

Table 3.2. Functional recovery of isolated rat hearts exposed to 20 minutes of global ischaemia (GI) followed by 30 minutes of reperfusion indicating the harmful consequences of this intervention. n=9.

Functional parameter	Pre-ischaemic	Post-ischaemic	Percentage recovery	Difference
Aortic output (ml/min)	48.00±2.51	12.72±4.18	25.24±10.76	P<0.0001
Cardiac output (ml/min)	64.44±2.53	24.11±4.96	37.52±9.57	P<0.0001
Total work (mW)	14.44±0.47	4.38±1.34	29.18±10.51	P<0.0001

PP2A during late ischaemia and early reperfusion

The primary reason why we used two experimental models (cell culture and isolated heart) to address the same question was to explore potential differences in these models, to utilize different techniques and to confirm our results. We therefore utilized our model of I/R injury in the isolated rat heart to characterize PP2A in exactly the same way as was done using SI in the H9c2 cells.

10, 15 and 20 minutes of global ischaemia

Figure 3.16 shows the effect of sustained ischaemia in the isolated rat heart on the levels of PP2A-A and –C. We made the very surprising observation that as ischaemia progressed the levels of both PP2A-C and –A increased so that it was significantly elevated above control at 20 minutes GI (PP2A-C: Control: 1.03±0.11 AU vs 20 min GI: 1.78±0.10 AU; P<0.05, PP2A-A: Control: 1.00±0.03 AU vs 20 min GI: 1.18±0.04 AU; P<0.05). This increase was especially evident in PP2A-C, to such a degree that at 20 minutes GI there was a significant increase in the amount of PP2A-C relative to PP2A-A (PP2A-C/A: Control: 1.02±0.07 AU vs 20 min GI: 1.62±0.15 AU; P<0.01).

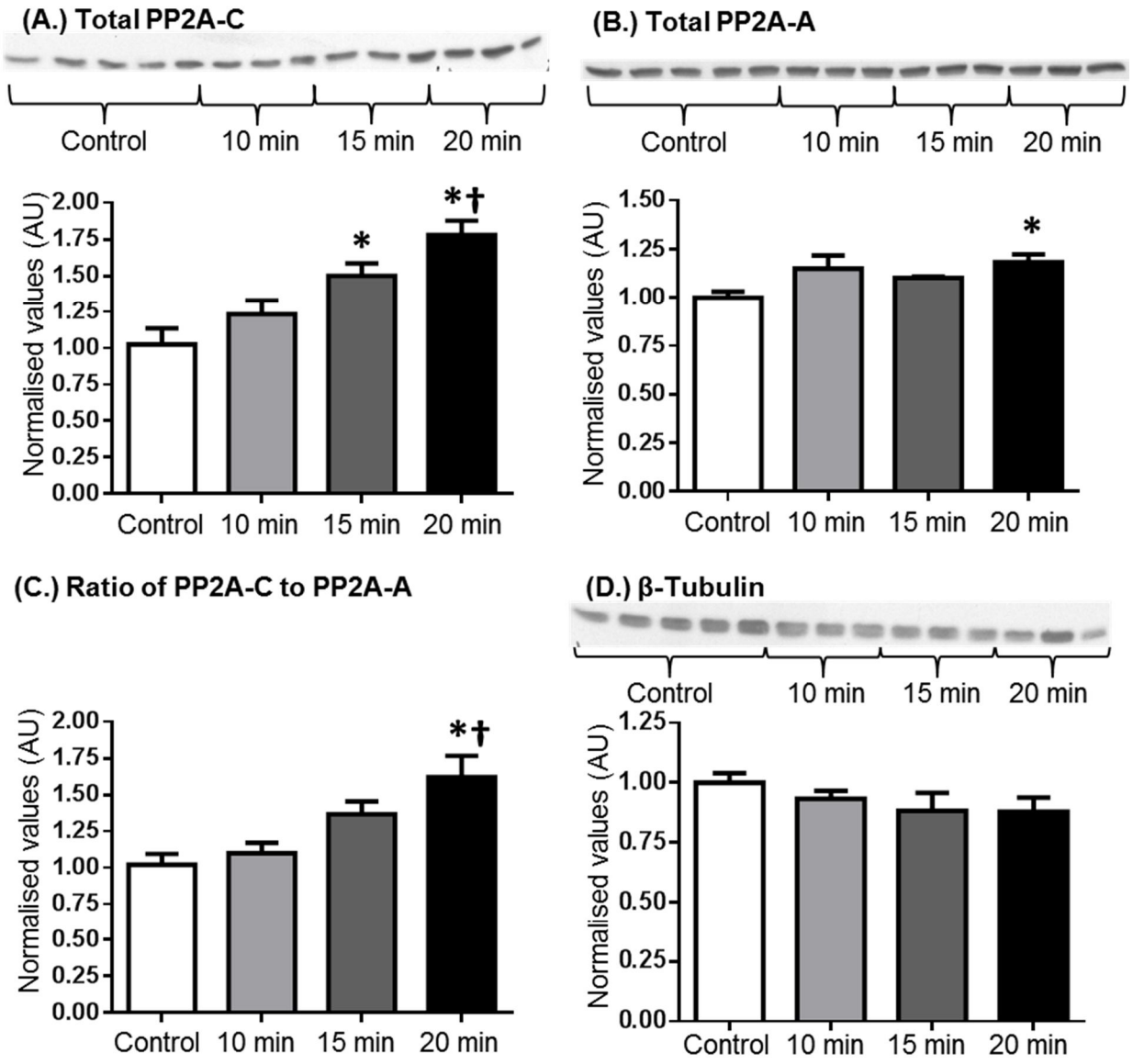


Figure 3.16. The effect of global ischaemia on PP2A in the rat heart. Sustained ischaemia is associated with an elevation in PP2A-C (A.) and PP2A-A (B.) levels, as well as an increase in the amount of PP2A-C relative to PP2A-A (C.)
*n=3-5; * P<0.05 vs control; †P<0.05 vs 10 minutes GI.*

Although there were no changes in phosphorylation and methylation relative to the cellular pool of PP2A-C (Figure 3.17), we observed an increase in absolute phosphorylated levels in late ischaemia (Control: 0.98±0.10 AU vs 20 min GI: 1.78±0.13 AU; P<0.05), as well as nonmethylation (Control: 1.00±0.19 AU vs 20 min GI: 2.30±0.14 AU; P<0.05). The failure of these significant changes to influence the relative degree of posttranslational modification of PP2A can obviously be ascribed to the concurrent increase in PP2A-C levels. This means that the proportion of the accumulated PP2A-C which is phosphorylated and not methylated remains unchanged. There is however an increase in the absolute cellular load of these modifications and it must therefore be of importance.

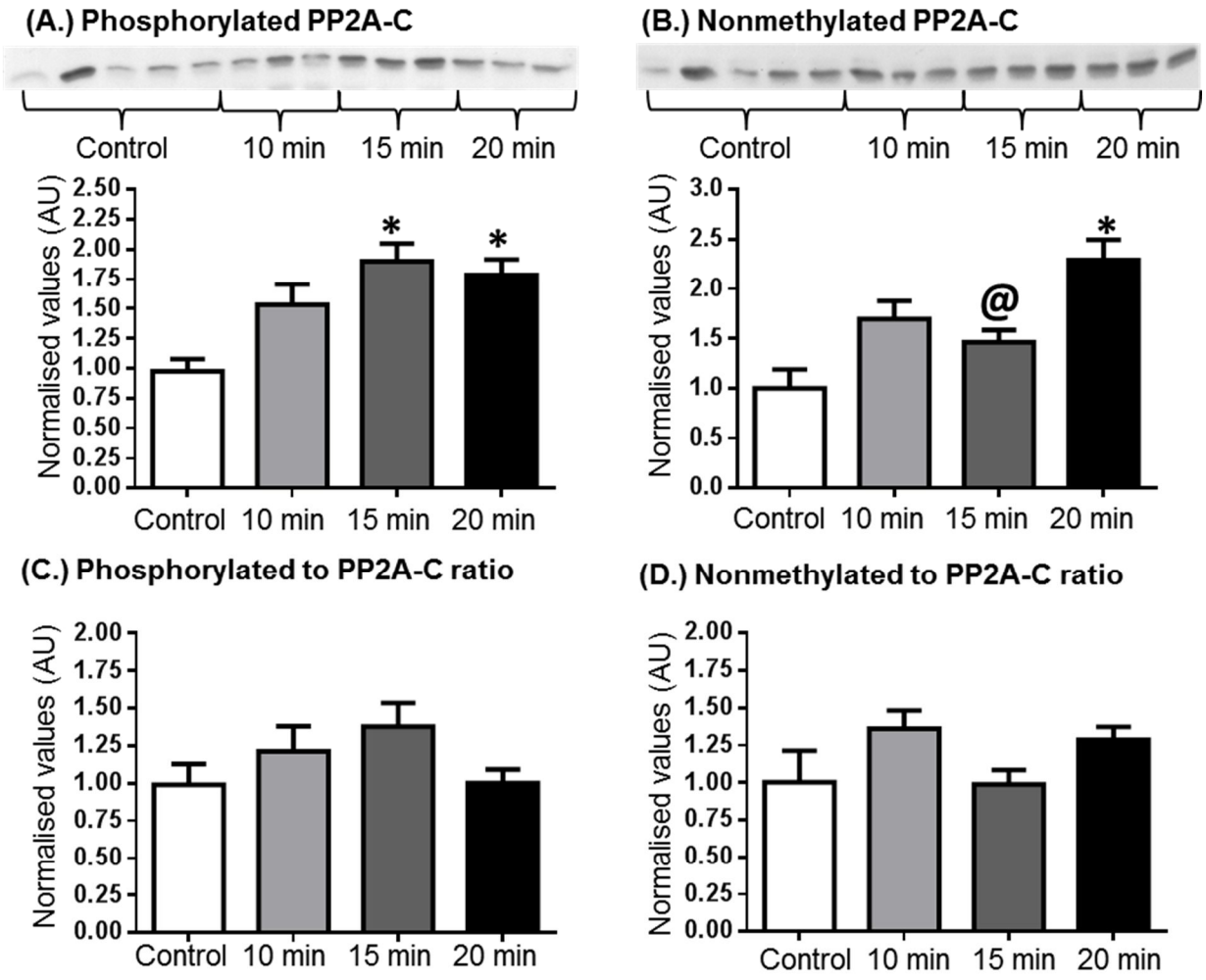


*Figure 3.17. The effect of global ischaemia on the posttranslational modification of PP2A-C. Although both phosphorylated and nonmethylated signals changed as ischaemia progressed, these changes were not significant relative to total PP2A-C. n=3-5; * P<0.05 vs control; @ P<0.05 vs 20 minutes GI.*

These results were so unexpected that we sought to confirm them by increasing the n-values. As will be described later in this thesis, we also did similar perfusion experiments later in the project in conjunction with pharmacological studies. In order to better assess the changes associated with 20 minutes GI we therefore decided to pool the normalized data from these different experiments for comparable timepoints (Figure 3.18). The result was a data set with a much larger n-value (10-16) and representing experiments spanning 4 years. This pooled data set confirmed our results: PP2A-C was elevated at 20 minutes ischaemia (Control: 1.00±0.04 AU vs 20 min GI: 1.30±0.10 AU; P<0.05), which was associated with an increase in PP2A-C/A (Control: 1.00±0.03 AU vs 20 min GI: 1.32±0.10 AU; P<0.01). Interestingly PP2A-A did not show an increase. The absolute levels of PP2A-C phosphorylation was also elevated (Control: 1.00±0.04 AU vs 20 min GI: 1.40±0.10 AU; P<0.01), as well as the levels of nonmethylation (Control: 1.00±0.07 AU vs 20 min GI: 1.76±0.19 AU; P<0.01). In fact, nonmethylation was also increased relative to total PP2A-C as well (Control: 1.00±0.08 AU vs 20 min GI: 1.30±0.04 AU; P<0.01).

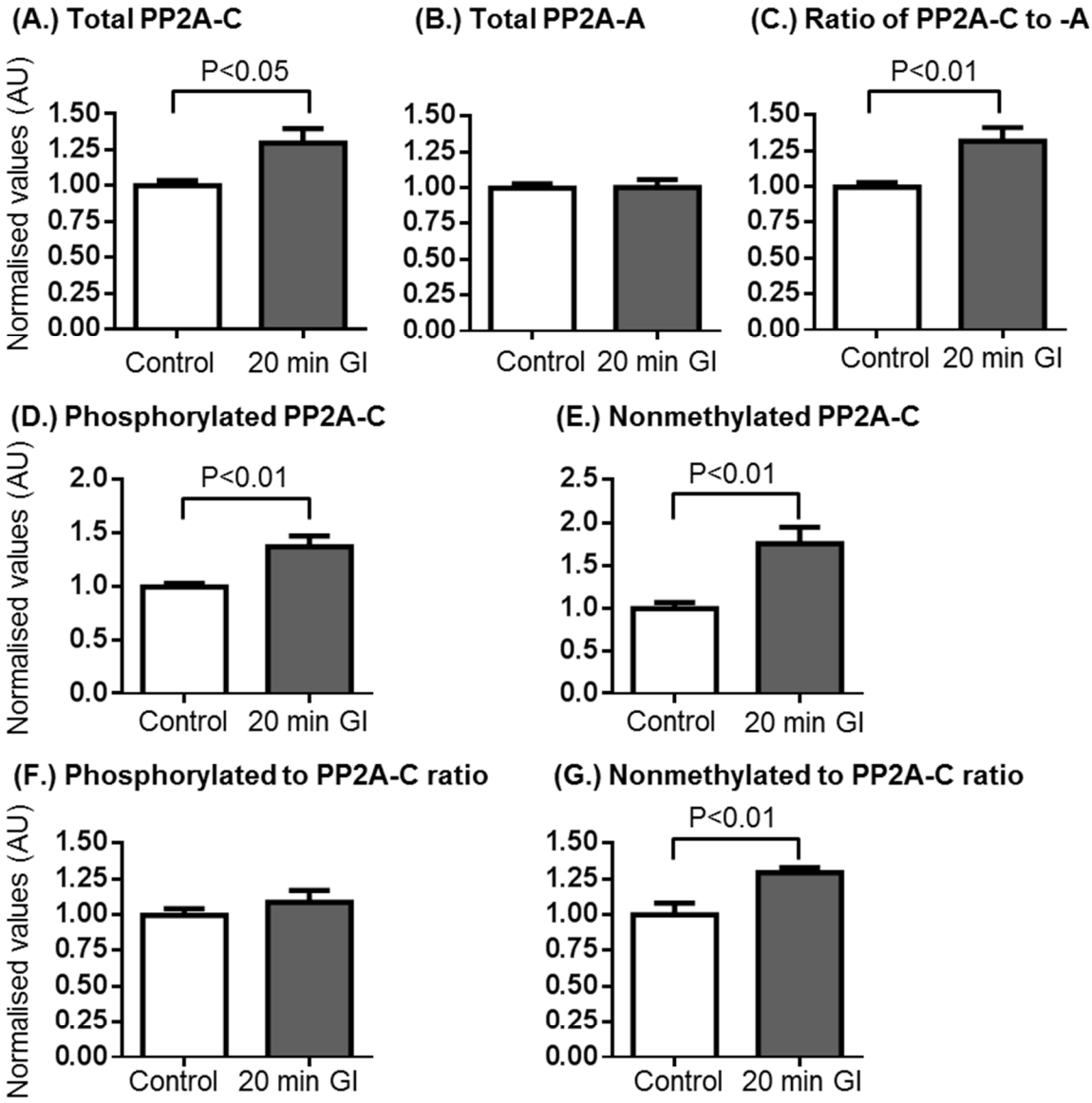


Figure 3.18. Combined data for PP2A expression and posttranslational modification at 20 minutes global ischaemia (GI). Combining the Western Blotting data from three different experiments confirmed the increase in the levels of PP2A-C at 20 minutes GI (A & C). This increase was associated with an increased phosphorylation and methylation of PP2A-C (D & E), although it is only nonmethylation which showed an increase relative to PP2A-C (G).
n=10-17.

5 and 10 minutes reperfusion

Just as surprising as our GI data, we observed an increase in total PP2A-C (Control: 1.00±0.06 AU vs 10 min Repf: 1.40±0.05 AU; P<0.05) and PP2A-A (Control: 1.00±0.02 AU vs 10 min Repf: 1.22±0.08 AU; P<0.05) levels at 10 minutes reperfusion (Figure 3.19). An observation that linked nicely with the increases observed at 20 minutes GI, but for the fact that both proteins were present at control levels at 5 minutes reperfusion – an observation which is very difficult to explain.

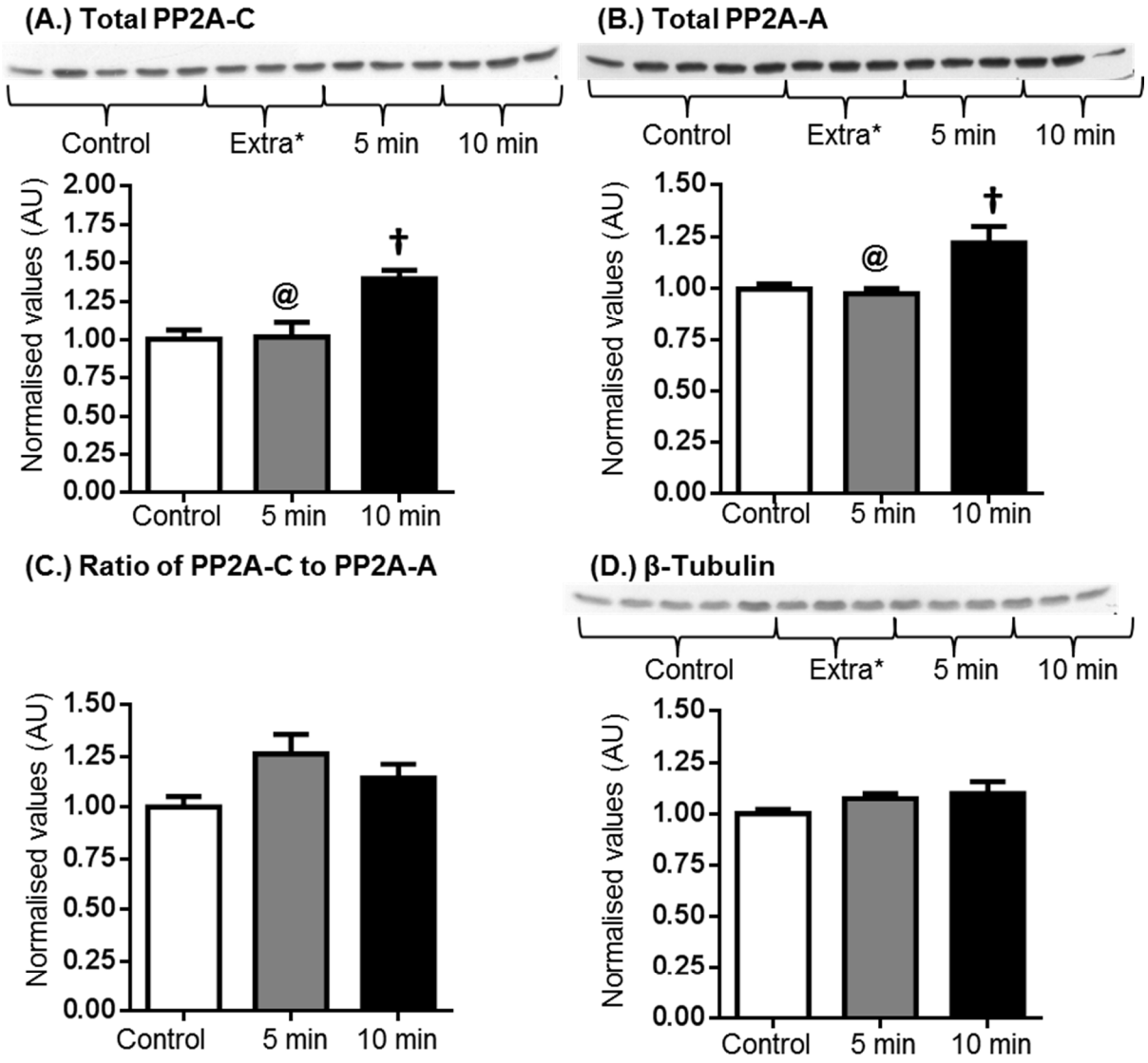


Figure 3.19. Total PP2A-C, PP2A-A and β-Tubulin during reperfusion. Ten minutes of reperfusion was associated with a significant increase in the levels of both PP2A-C and –A.
** These three hearts served as extra controls which were eventually not included in the analysis.*
n=3-5; † P<0.05 vs Control; @ P<0.05 vs. 10 minutes reperfusion.

Figure 3.20 shows the posttranslational modifications of PP2A-C at the onset of reperfusion in the original experiment. Five minutes reperfusion was associated with a robust increase in both phosphorylation (absolute levels: Control: 1.00±0.11 AU vs 5 min Repf: 1.52±0.09 AU; P<0.05; relative to PP2A-C: Control: 1.00±0.11 AU vs 5 min Repf: 1.86±0.16 AU, P<0.05) and nonmethylation (absolute levels: Control: 1.00±0.11 AU vs 5 min Repf: 1.67±0.11 AU; P<0.05, relative to PP2A-C: Control: 1.00±0.13 AU vs 5 min Repf: 2.00±0.17 AU; P<0.05).

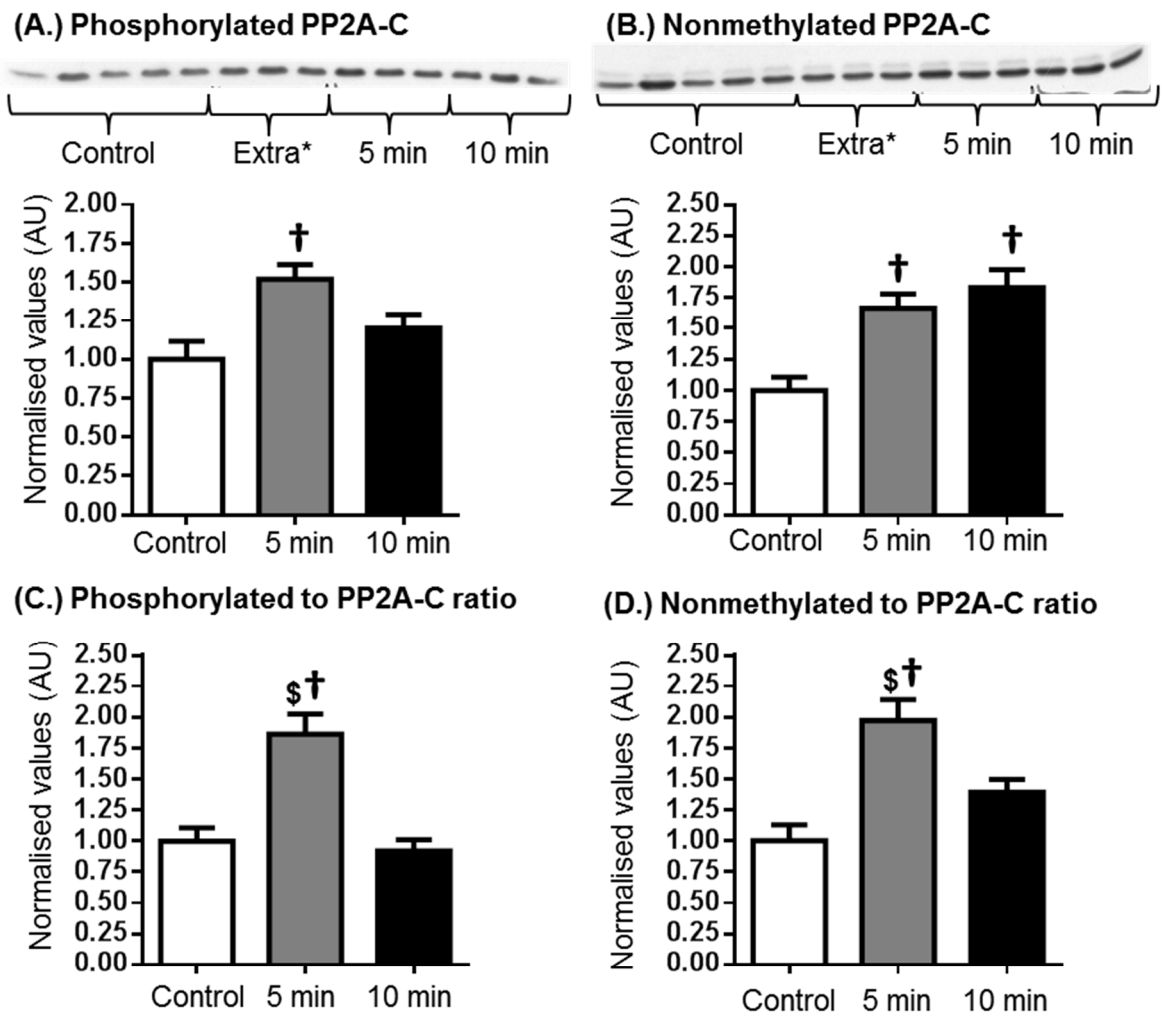


Figure 3.20. The effect of reperfusion on the post-translational modification of PP2A-C. Initial reperfusion was associated with an increase in both phosphorylation (A & C), as well as nonmethylation (B & D). Except for absolute nonmethylation (B.) these changes disappeared at 10 minutes reperfusion.
** These three hearts served as extra controls which were eventually not included in the analysis.*
n=3-5; † P<0.05 vs control; $ P<0.05 vs 10 minutes reperfusion.

The repetition of these reperfusion experiments in combination with later pharmacological studies however gave us the opportunity to pool our data in the same way as was done for 20 minutes GI. These results showed a much different picture regarding the fluctuations in total protein levels during the first 10 minutes of reperfusion. At 5 minutes reperfusion (Figure 3.21) there was a slight decrease in PP2A-A (Control: 1.00±0.02 AU vs 5 min Repf: 0.85±0.05 AU; P<0.01), with an associated increase in the ratio of PP2A-C to –A (Control: 1.00±0.03 AU vs 10 min Repf: 1.13±0.06 AU; P<0.05). At 10 minutes reperfusion the pooled data showed no increase in PP2A-C or –A (Figure 3.22). This would indicate that following ischaemia there is a rectification in the levels of PP2A-C and –A back to control levels. The slight reduction in PP2A-A seen at 5 minutes reperfusion was possibly due to washout of cellular protein from damaged and dead cells following I/R. This might also explain the reduced PP2A-C and –A relative to 10 minutes reperfusion in the original data set (Figure 3.19).

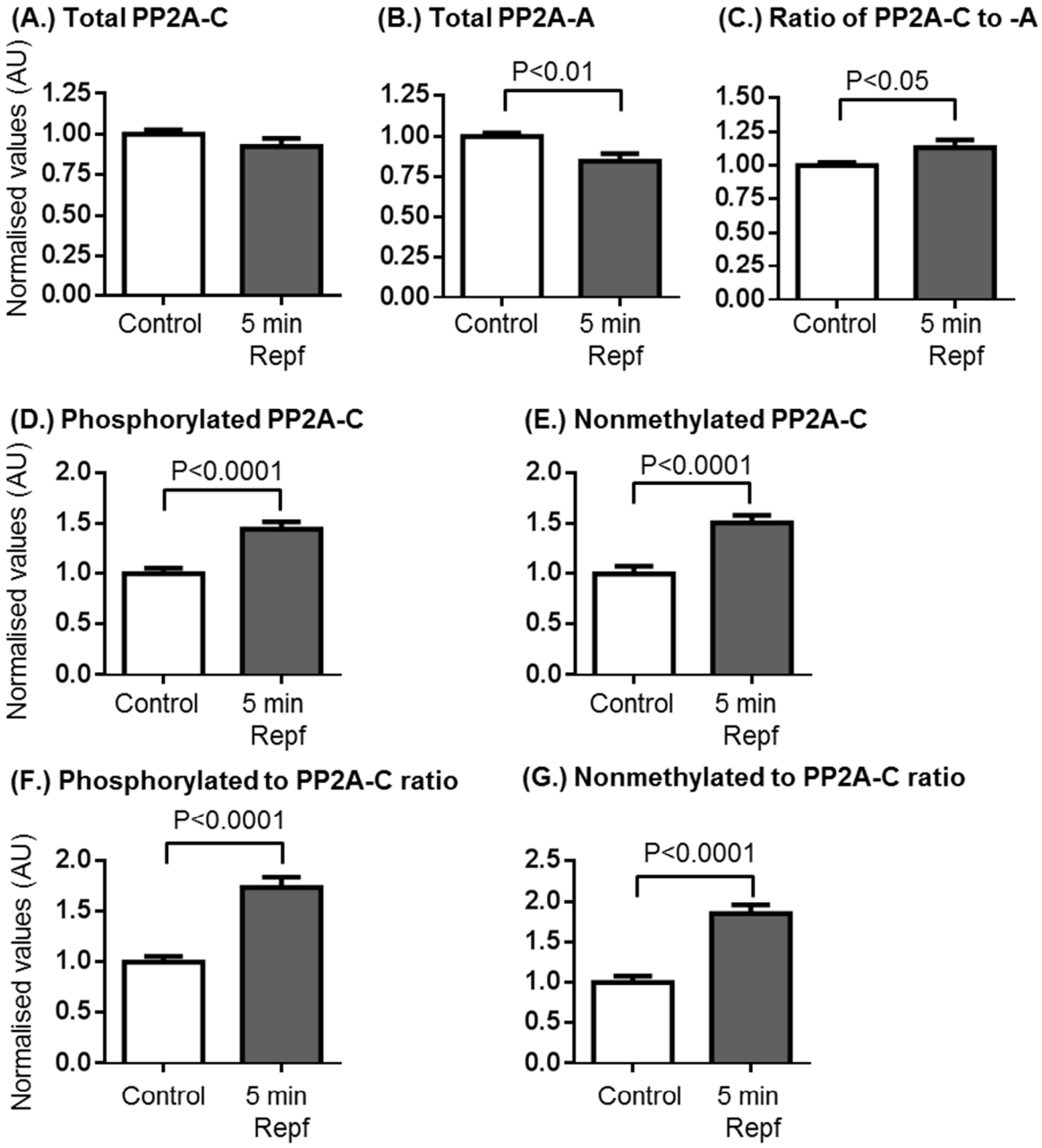


Figure 3.21. Combined data for PP2A expression and posttranslational modification at 5 minutes reperfusion. Both the degree of phosphorylation (D & F), as well as nonmethylation (E & G), of PP2A-C increased. There was however no change in the levels of PP2A-C (A), while PP2A-A was reduced (B). This loss of PP2A-A caused a shift in the ratio between PP2A-C and PP2A-A (C).
n=11-13.

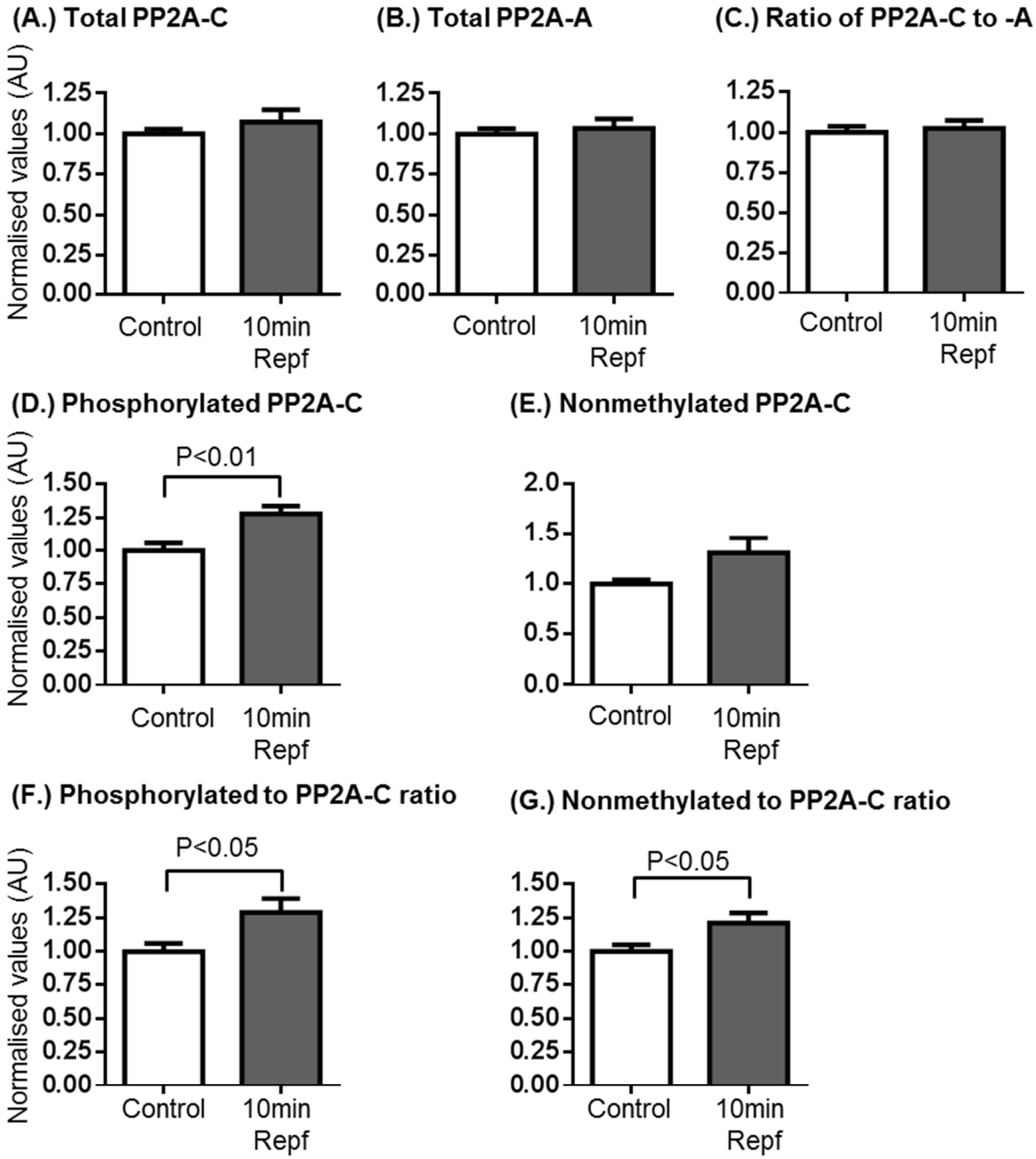


Figure 3.22. Combined data for PP2A expression and posttranslational modification at 10 minutes reperfusion. The level of phosphorylation of PP2A-C showed an increase (D & F), as well as the relative degree of nonmethylation (G). All other parameters remained unchanged.
n=11-14.

Interestingly, despite the loss of differences in total protein in the combined data set, these posttranslational modifications are confirmed and even more evident in the larger group (Figures 3.21 and 3.22). Five minutes reperfusion was associated with an increase in the relative degree of both phosphorylation (Control: 1.00±0.06 AU vs 5 min Repf: 1.74±0.12 AU; P<0.0001) and nonmethylation (Control: 1.00±0.08 AU vs 5 min Repf: 1.85±0.11 AU; P<0.0001), with 10 minutes reperfusion following exactly the same trend (relative phosphorylation: Control: 1.00±0.06 AU vs 10 min Repf: 1.30±0.10 AU; P<0.05, and relative nonmethylation: Control: 1.00±0.05 AU vs 10 min

Repf: 1.21±0.07 AU; P<0.05). Our data therefore clearly illustrates a very robust pattern of phosphorylation and demethylation of PP2A at the onset of reperfusion.

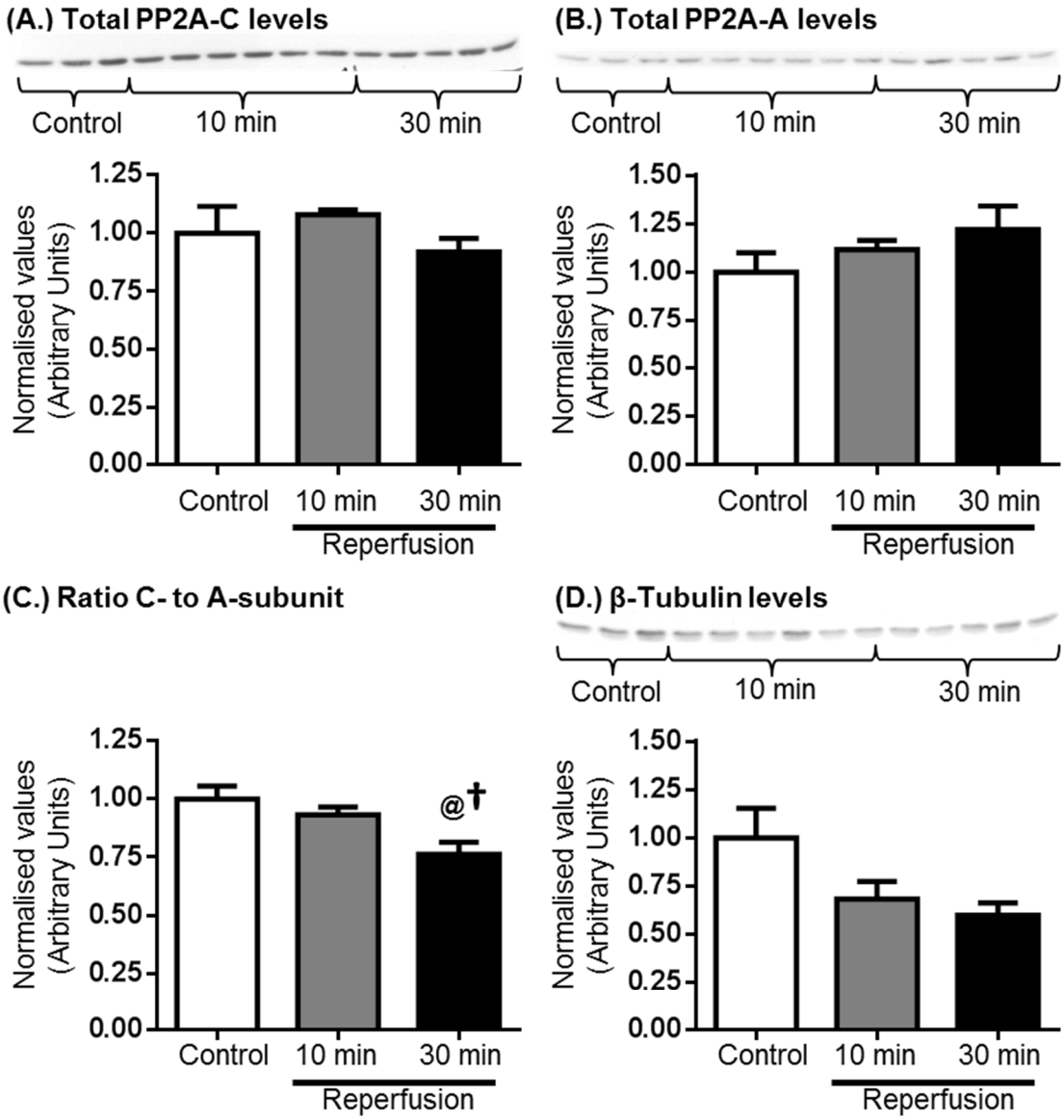


Figure 3.23. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 30 minutes reperfusion following 20 minutes of global ischaemia. Although at this stage of reperfusion there were no changes in total PP2A-C or –A (A & B), the ratio between the two has shifted so that there is relatively more PP2A-A than PP2A-C. n=3-6; @ P<0.05 vs 10 minutes reperfusion, † P<0.05 vs control.

Having observed these changes in early reperfusion, raised the question as to the duration of these effects; will they still be evident at 30 minutes reperfusion? As shown on Figures 3.23 and 3.24 these early reperfusion events were all absent by late reperfusion. In fact, at 30 minutes reperfusion there was a reduction in the ratio of PP2A-C/A (Control: 1.00±0.06 AU, and 10 min Repf: 0.93±0.03 AU vs 30 min Repf: 0.76±0.05 AU; P<0.05), indicating an excess PP2A-A for the available PP2A-C. Thirty minutes of reperfusion was also associated with a reduction in absolute phosphorylation of PP2A in comparison with 10 minutes reperfusion (10 min Repf: 1.00±0.12 AU

vs 30 min Repf; P<0.05), an effect not seen relative to total PP2A-C. It therefore seems as if all the measured parameters are nearing normal values again at 30 minutes reperfusion.

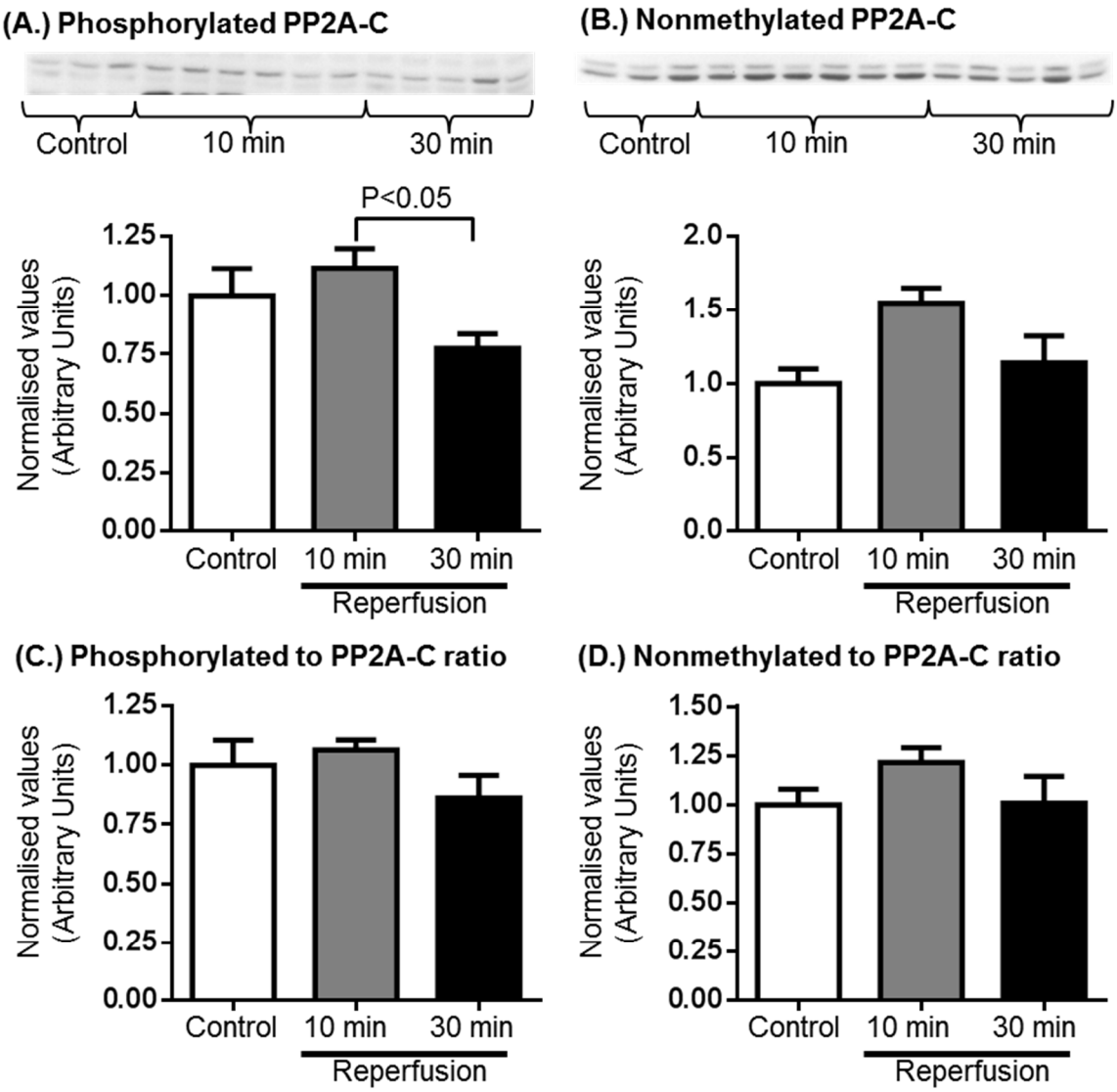


Figure 3.24. Phosphorylation (A & C) and methylation (B & D) of PP2A-C following 30 minutes of reperfusion. There was a reduction in the absolute levels of phosphorylation of PP2A-C, although not relative to total PP2A-C.
n=2-6; one representative blot of the results is shown.

Our results showed that sustained ischaemia and reperfusion exerted an effect on PP2A-C and PP2A-A levels, as well as the levels of phosphorylation and methylation of PP2A-C. Taking into account the fact that the regulation of PP2A is to a large degree dependent on the cellular localization of the enzyme, the question arose as to what is happening with regards to PP2A in different cellular fractions.

The cellular distribution and posttranslational modification of PP2A during ischaemia and reperfusion.

To answer this question, tissues from control hearts and hearts exposed to different durations of ischaemia and reperfusion were lyzed and fractionated using differential centrifugation into three fractions: nuclear, cytosolic and membrane. Each fraction was then analysed using Western blotting for the same targets as described for the unfractionated samples.

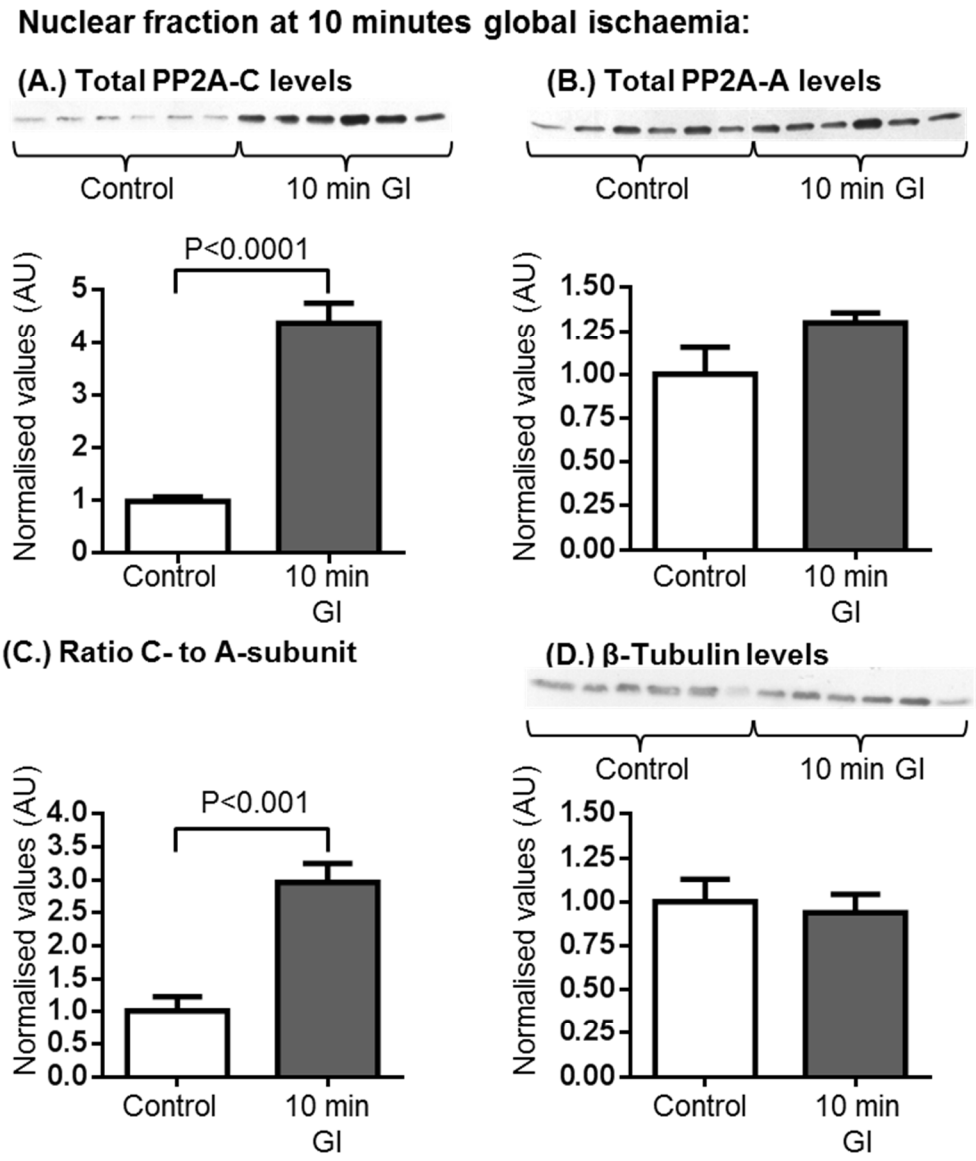


Figure 3.25. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 10 minutes global ichaemia (GI) as measured in the nuclear fraction. There was a pronounced accumulation of PP2A-C in the nuclear fraction, while PP2A-A did not follow this same trend. n=5-6.

10 minutes global ischaemia

At 10 minutes GI there was a pronounced increase in the levels of PP2A-C in the nuclear fraction (Control: 0.98±0.08 AU vs 10 min GI: 4.38±0.37 AU; P<0.0001), with a concomitant increase in PP2A-C/A (Control: 1.02±0.21 AU vs 10 min GI: 2.97±0.28 AU; P<0.001) indicating an extreme

accumulation of PP2A-C in the nuclear fraction in the absence of changes in PP2A-A; i.e. there was no accumulation of either the dimeric or trimeric forms of the enzyme (Figure 3.25).

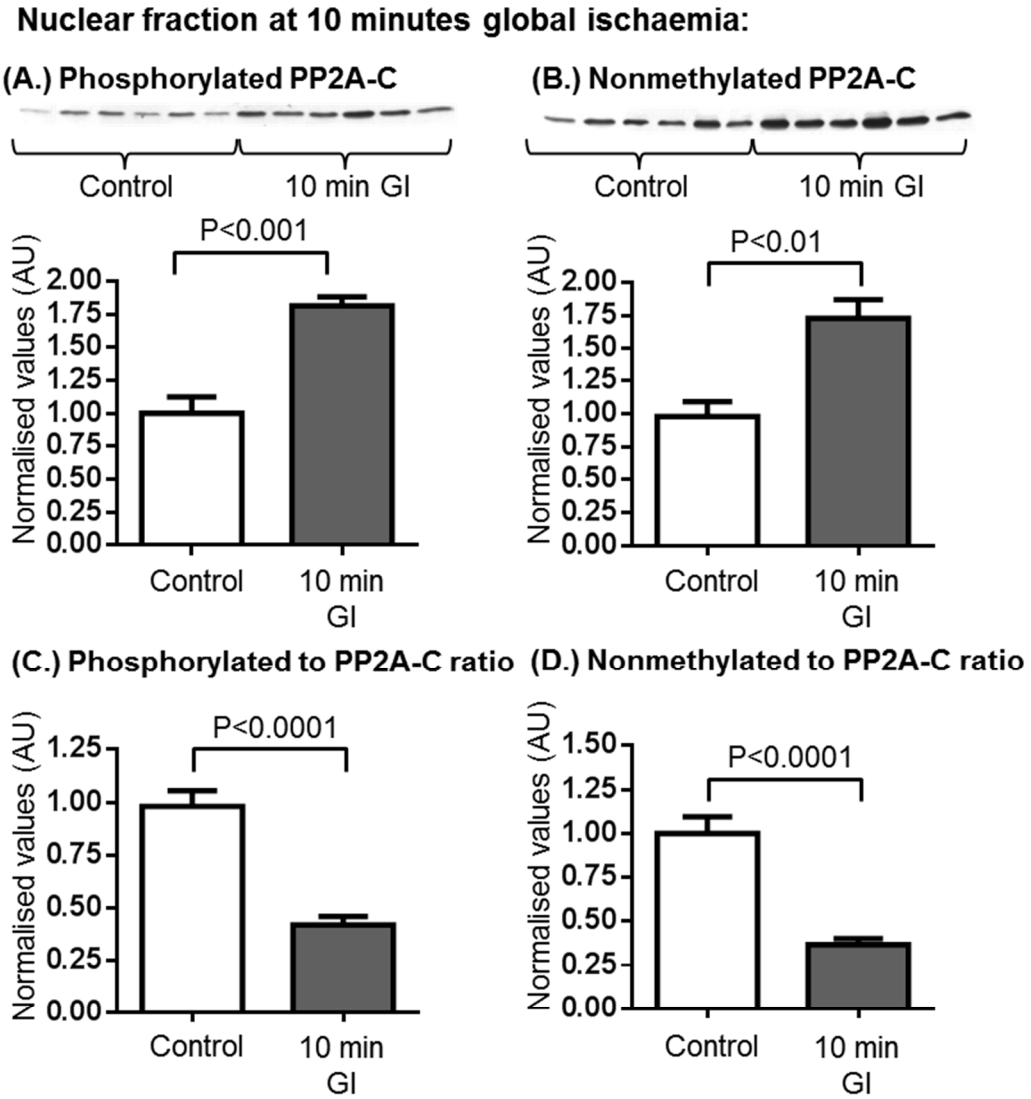


Figure 3.26. Phosphorylation and methylation of PP2A-C at 10 minutes global ischaemia (GI) in the nuclear fraction. Although the absolute levels of both phosphorylated and nonmethylated PP2A-C were increased (A & B), there was such a massive accumulation of PP2A-C in the nuclear fraction that the relative values were actually reduced (C & D).
n=5-6.

Although the absolute levels of phosphorylation (Control: 1.00±0.12 AU vs 10 min GI: 1.82±0.07 AU; P<0.001) and nonmethylation (Control: 0.98±0.11 AU vs 10 min GI: 1.73±0.14 AU; P<0.01) increased in the nucleus, the elevation of PP2A-C was so great that the relative values were actually reduced (Phosphorylation: Control: 0.98±0.08 AU vs 10 min GI: 0.42±0.04 AU; P<0.0001; and nonmethylation: Control: 1.00±0.10 AU vs 10 min GI: 0.37±0.03 AU; P<0.0001). This means that the PP2A-C in the nucleus was not only not associated with PP2A-A, but it was also unphosphorylated and methylated (Figure 3.26).

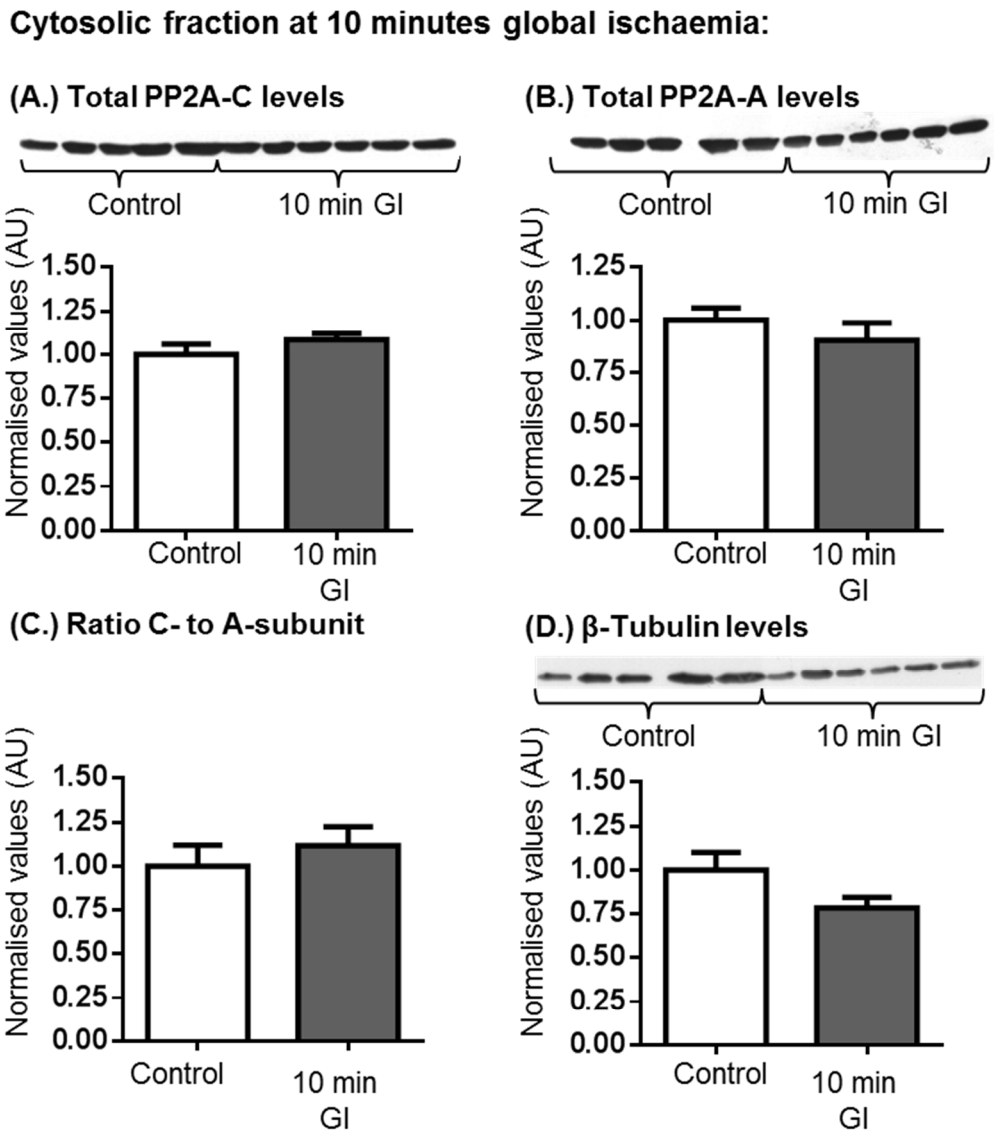


Figure 3.27. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 10 minutes global ichaemia (GI) as measured in the cytosolic fraction. Ischaemia failed to elicit any changes in any of the primary targets of this study.
n=3-6.

These fluctuations in the nuclear fraction occurred in the absence of any changes in the levels of PP2A-C in the cytosolic and membrane fractions (Figures 3.27 – 3.30). In fact the only significant change was in the relative methylation of PP2A in the membrane fraction (Control: 1.00±0.07 AU vs 10 min GI: 1.27±0.07 AU; $P<0.05$).

Cytosolic fraction at 10 minutes global ischaemia:

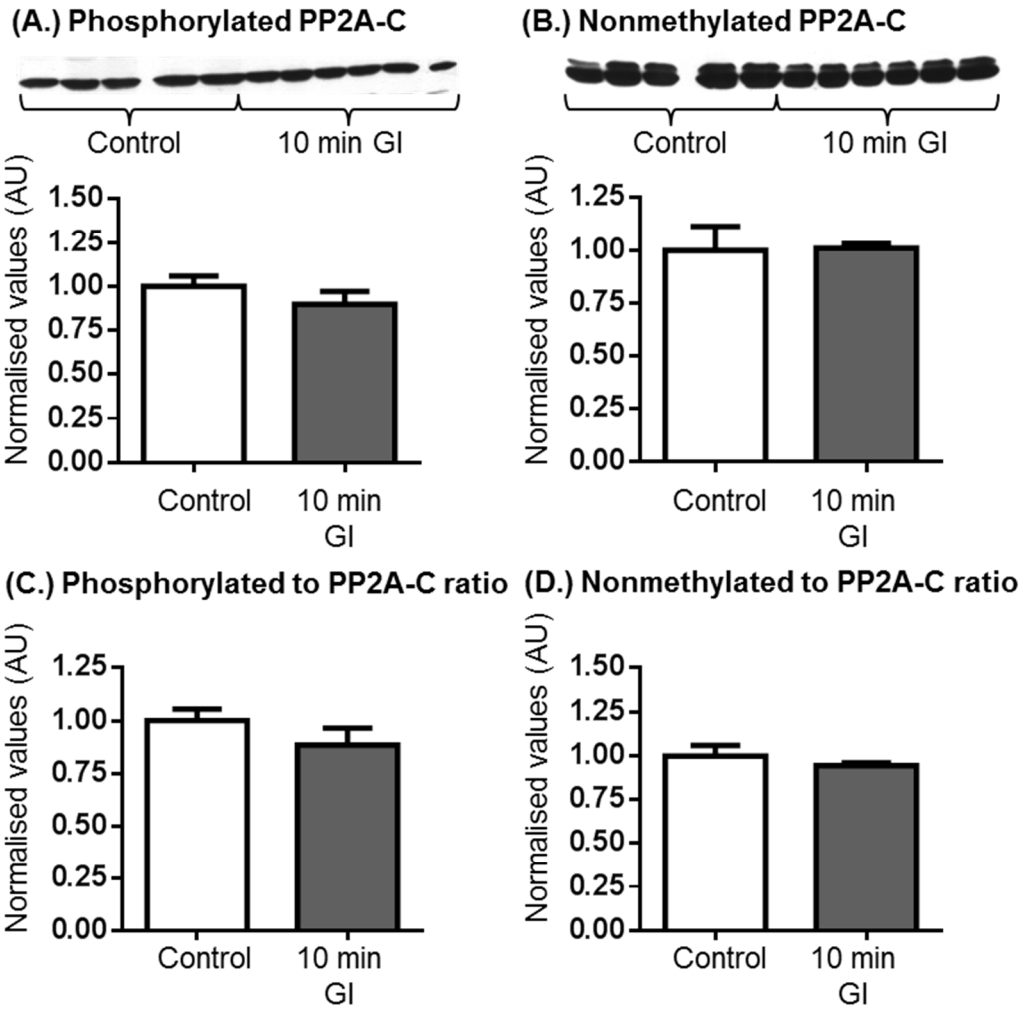


Figure 3.28. Phosphorylation and methylation of PP2A-C at 10 minutes global ischaemia (GI) in the cytosolic fraction. There were no differences with regards to any of these modifications.
n=3-6.

Membrane fraction at 10 minutes global ischaemia:

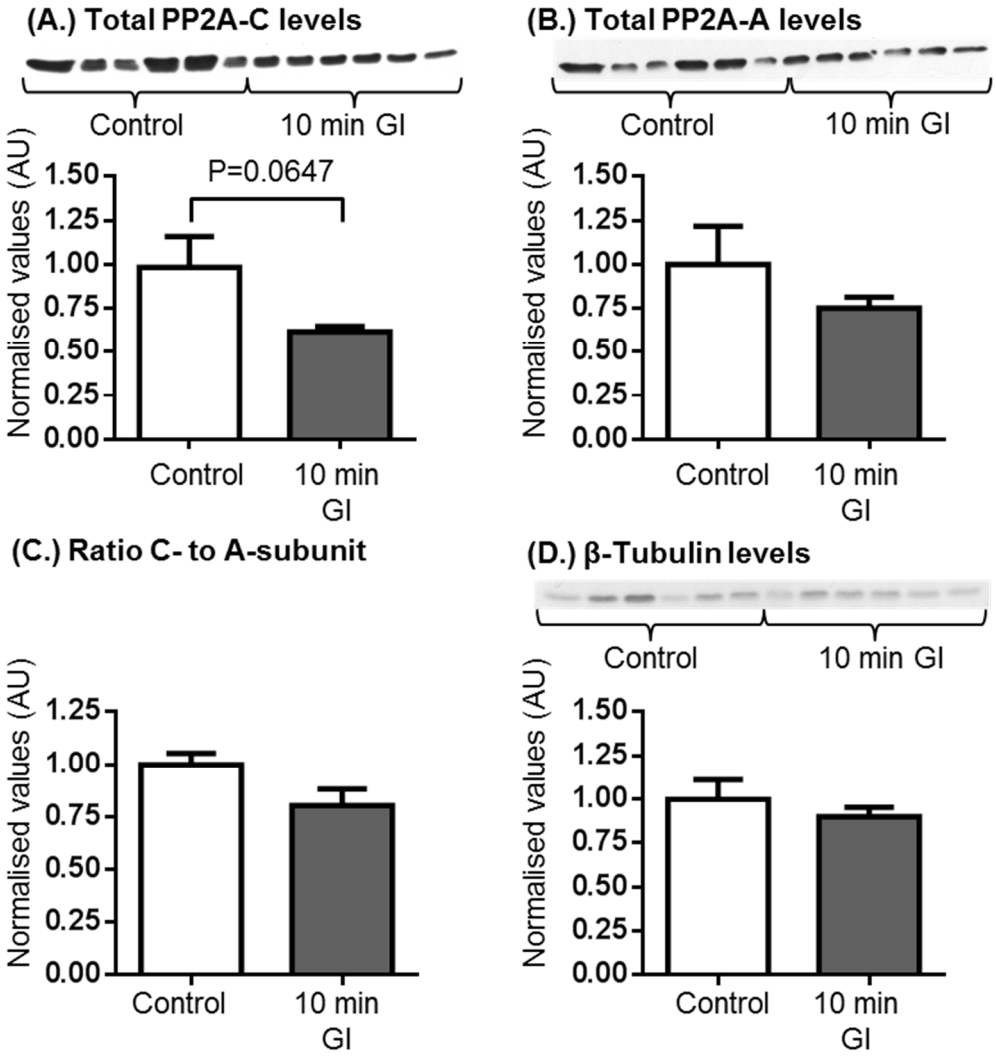


Figure 3.29. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 10 minutes global ichaemia (GI) as measured in the membrane fraction. n=6.

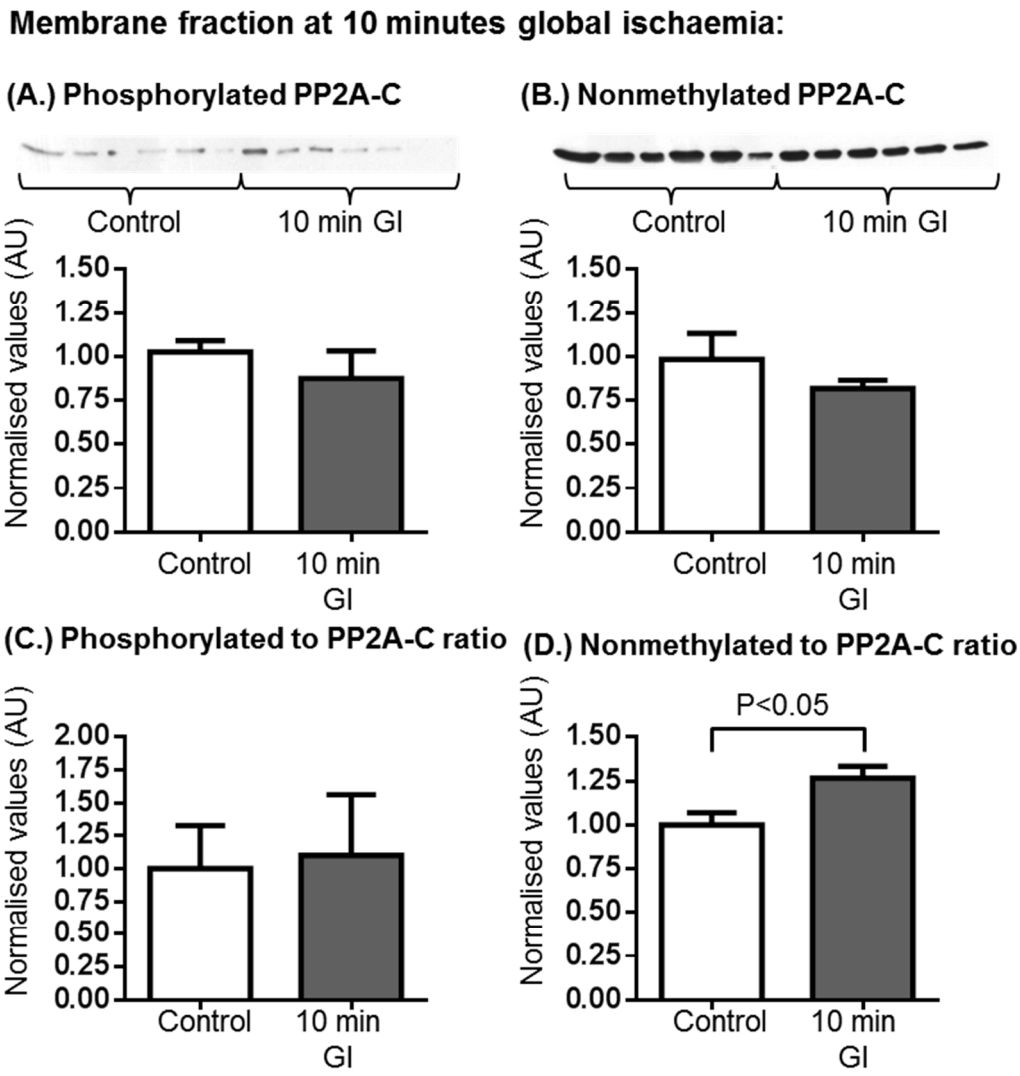


Figure 3.30. Phosphorylation and methylation of PP2A-C at 10 minutes global ischaemia (GI) in the membrane fraction. There was an increase in the nonmethylation of PP2A-C relative to total at this time point. n=4-6.

The question therefore arises where does the PP2A-C accumulating in the nucleus comes from? One possible explanation for why we did not see a reduction in any of the other fractions relates to the relative amounts of PP2A-C in the three fractions. Of the three fractions, under normal control conditions the nucleus contains by far the least amount of PP2A-C (pixels expressed relative to the amount of protein loaded onto SDS-PAGE: nucleus: 199.4±52.7 pixels/µg vs. cytosolic: 4381.0±319.2 pixels/µg and membrane: 2710.0±472.2 pixels/µg; if expressed relative to the nucleus: 1.00±0.3 AU vs. cytosolic: 22.0±1.6 AU vs membrane: 13.6±2.4 AU, n=4; P<0.001). The implication of this is that a small reduction in the other fractions could lead to a massive increase in the relatively small pool present in the nucleus. Keeping this in mind, it is noteworthy that 10 minutes GI was associated with a borderline significant reduction in PP2A-C levels in the membrane fraction (Figure 3.29: Control: 0.98±0.17 AU vs. 10 min GI: 0.62±0.03 AU; P=0.0647), thereby identifying the membrane as a possible contributor to the accumulated PP2A-C pool in the nucleus.

15 minutes global ischaemia

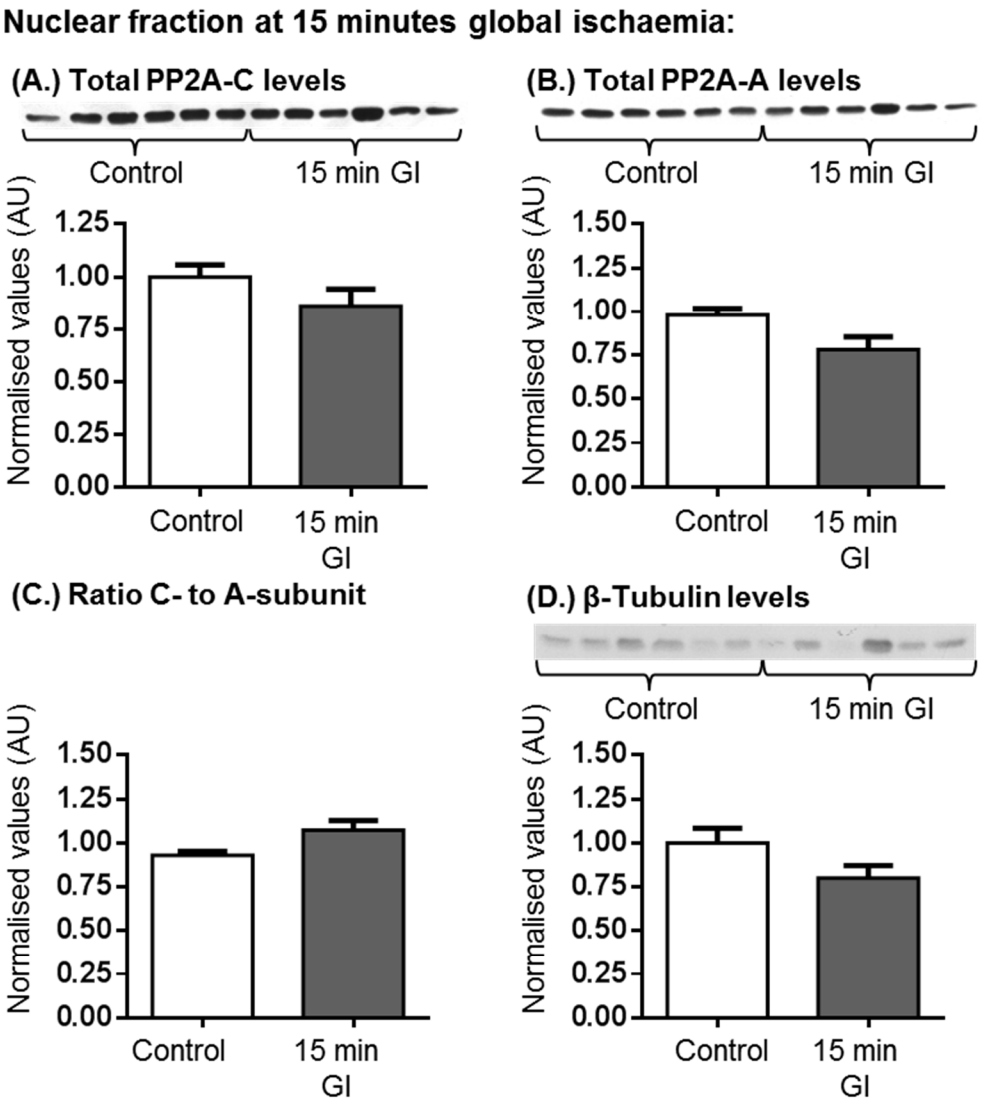


Figure 3.31. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 15 minutes global ichaemia (GI) as measured in the nuclear fraction. No changes in the levels of any of the target proteins were measured. n=5-6.

Only 5 minutes later the large increase of PP2A-C observed in the nucleus at 10 minutes GI had disappeared (Figure 3.31). Phosphorylation and methylation of PP2A also show no deviation from control values at this time (Figure 3.32).

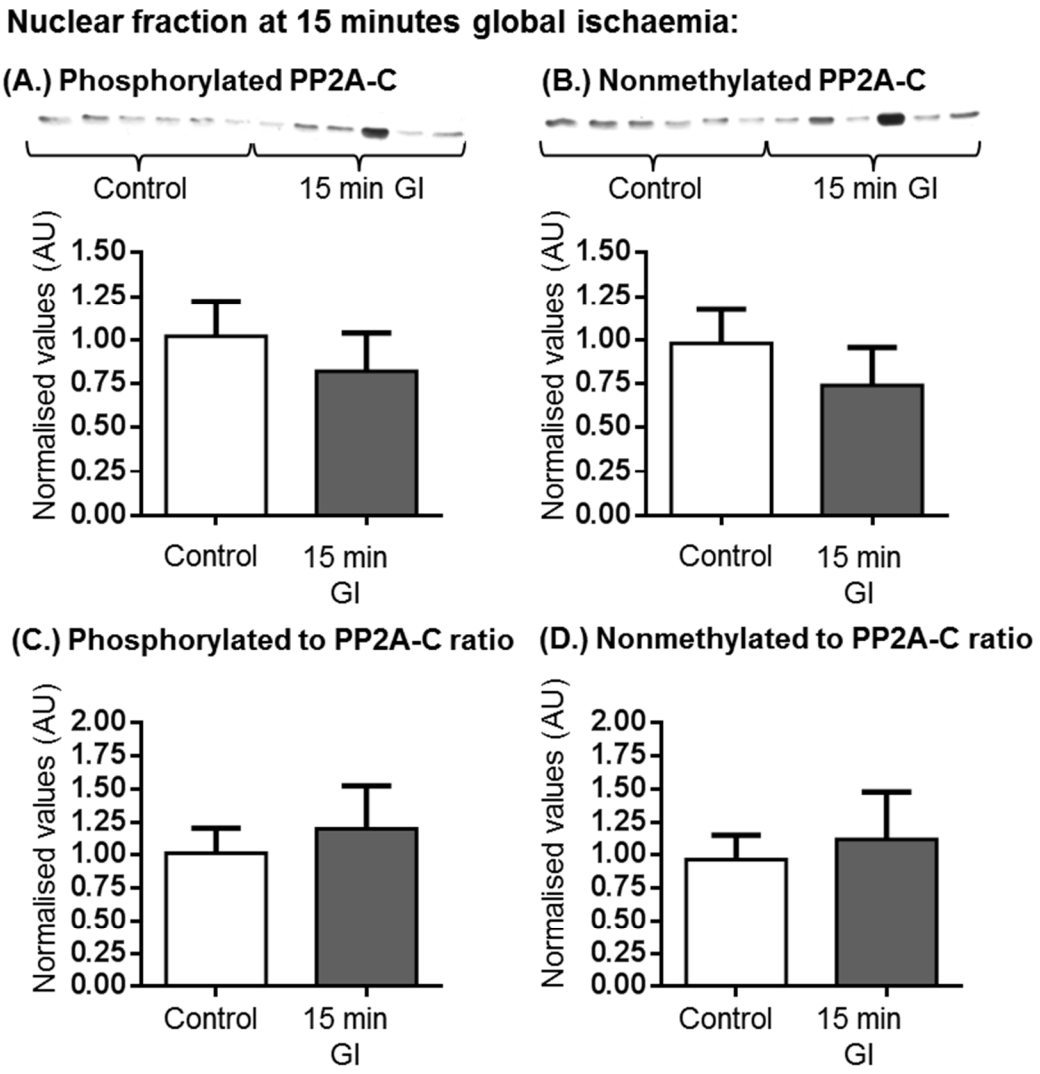


Figure 3.32. Phosphorylation and methylation of PP2A-C at 15 minutes global ischaemia (GI) in the nuclear fraction. No alterations were associated with this time period.
n=5.

In contrast to this inactivity in the nuclear fraction there is a significant increase in the levels of PP2A-C in the cytosolic fraction (Control: 1.02±0.03 AU vs 15 min GI: 1.22±0.04 AU; $P<0.01$), although not enough to cause a shift in the PP2A-C/A value (Figure 3.33). This increase in PP2A-C in the cytosol is associated with a reduction in phosphorylation (absolute phosphorylated: Control: 1.00±0.18 AU vs 15 min GI: 0.38±0.11 AU; $P<0.05$; and phosphorylated relative to total: Control: 1.02±0.18 AU vs 15 min GI: 0.30±0.08 AU; $P<0.01$), implying that the PP2A-C present in the cytosol is predominantly active (Figure 3.34).

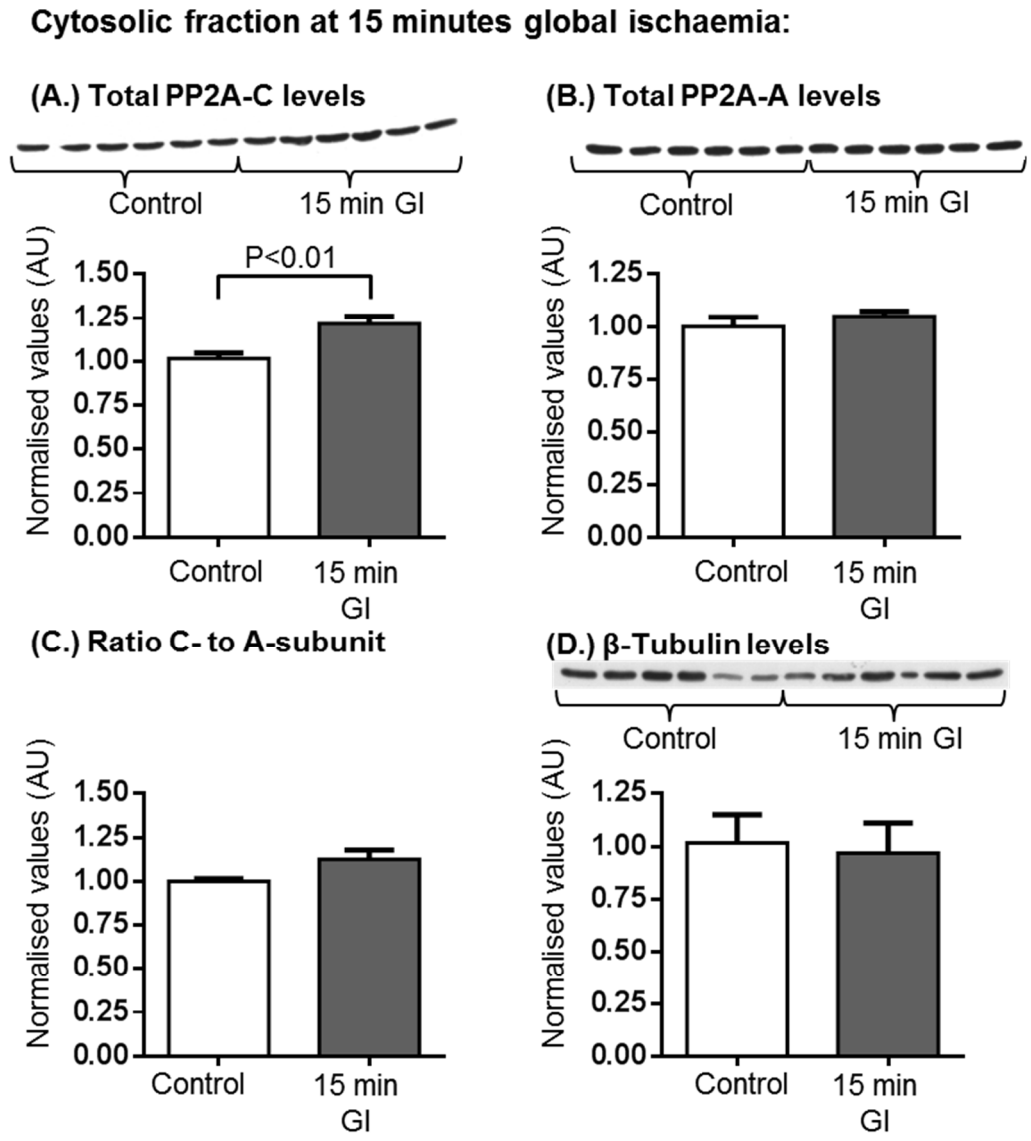


Figure 3.33. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 15 minutes global ichaemia (GI) as measured in the cytosolic fraction. PP2A-C showed an increase at this time point.
n=6.

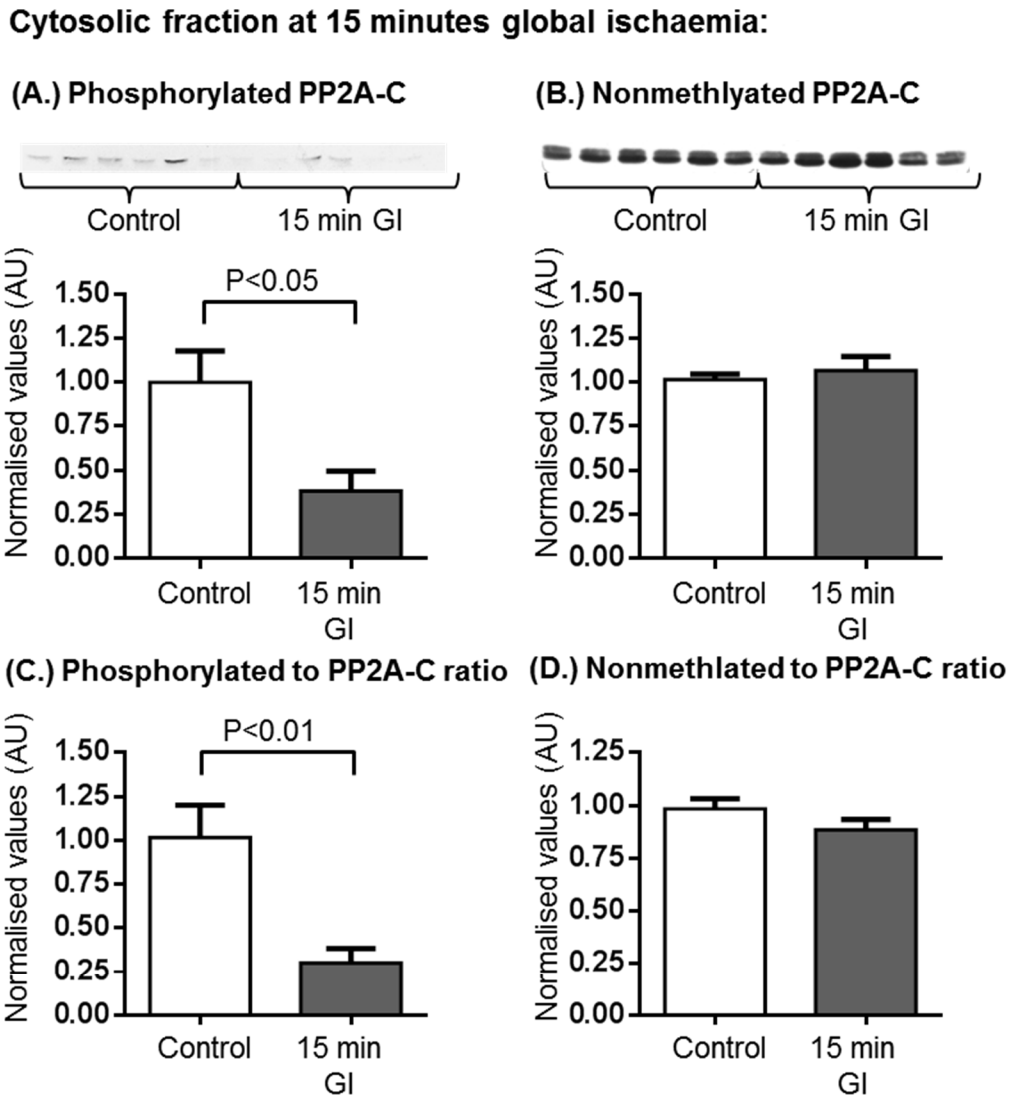


Figure 3.34. Phosphorylation and methylation of PP2A-C at 15 minutes global ischaemia (GI) in the cytosolic fraction. Ischaemia induced a profound reduction in the phosphorylation of PP2A-C, both in absolute levels as well as relative to total PP2A-C.
n=5-6.

In the membrane fraction (Figure 3.35) we unexpectedly saw a significantly higher signal for β-Tubulin in the ischaemic group than in the control group (Control: 1.00±0.11 AU vs 15 min GI: 1.45±0.05 AU; P<0.01). The explanation for this anomaly is unknown and might simply be due to a miscalculation concerning protein content in the final lysates. Irrespective of the reason, it necessitated us to recalculate our total PP2A-A and –C data by expressing them relative to β-Tubulin (Figure 3.36). This adjustment of the data revealed a borderline significant reduction in PP2A-A (Control: 1.07±0.30 AU vs 15 min GI: 0.46±0.03 AU; P=0.09) which contributed to a significant increase in the ratio of PP2A-C to –A (Control: 1.04±0.07 AU vs 15 min GI: 1.84±0.15 AU; P<0.01).

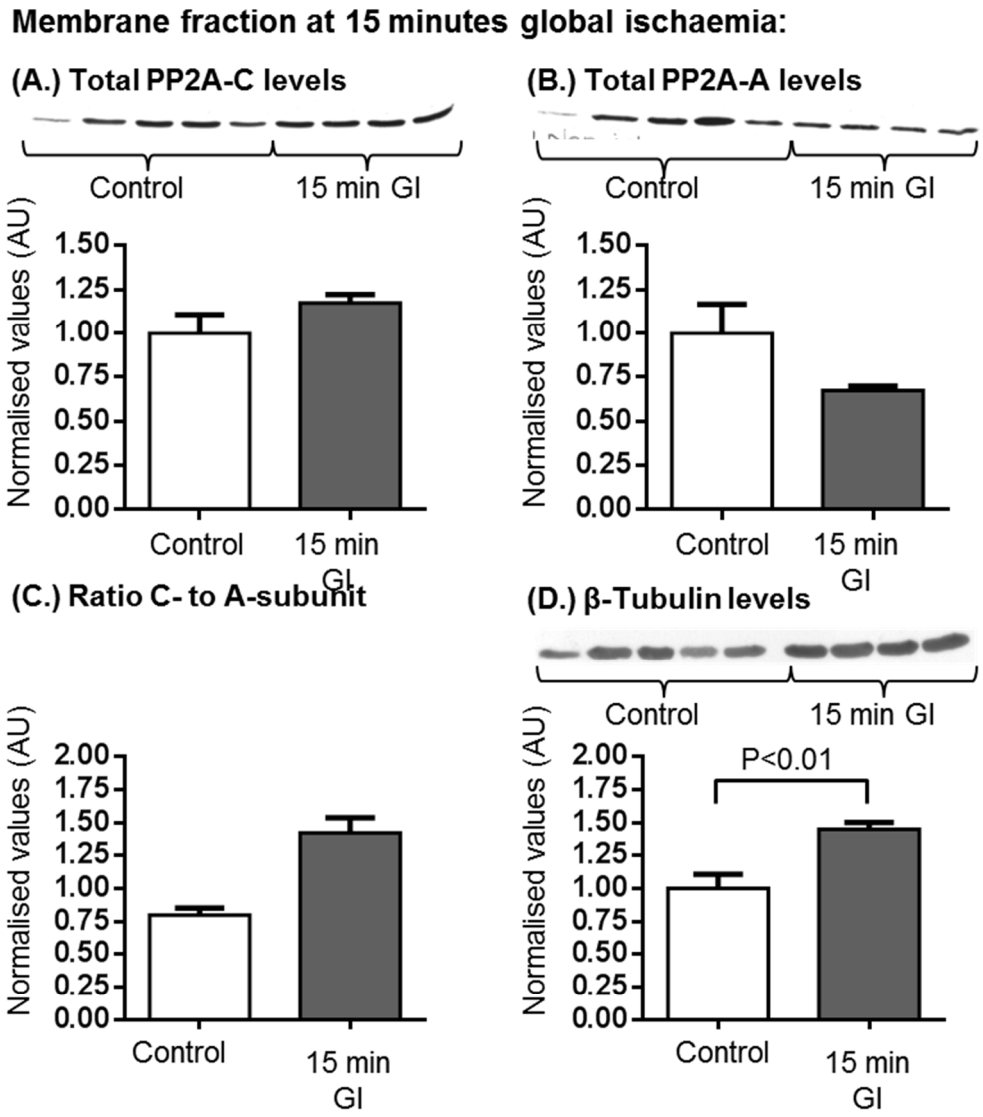


Figure 3.35. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 15 minutes global ischaemia (GI) as measured in the membrane fraction. Although there were no differences with regards to PP2A-C and PP2A-A; β-Tubulin showed an increase in the ischaemic tissue.
n=4.

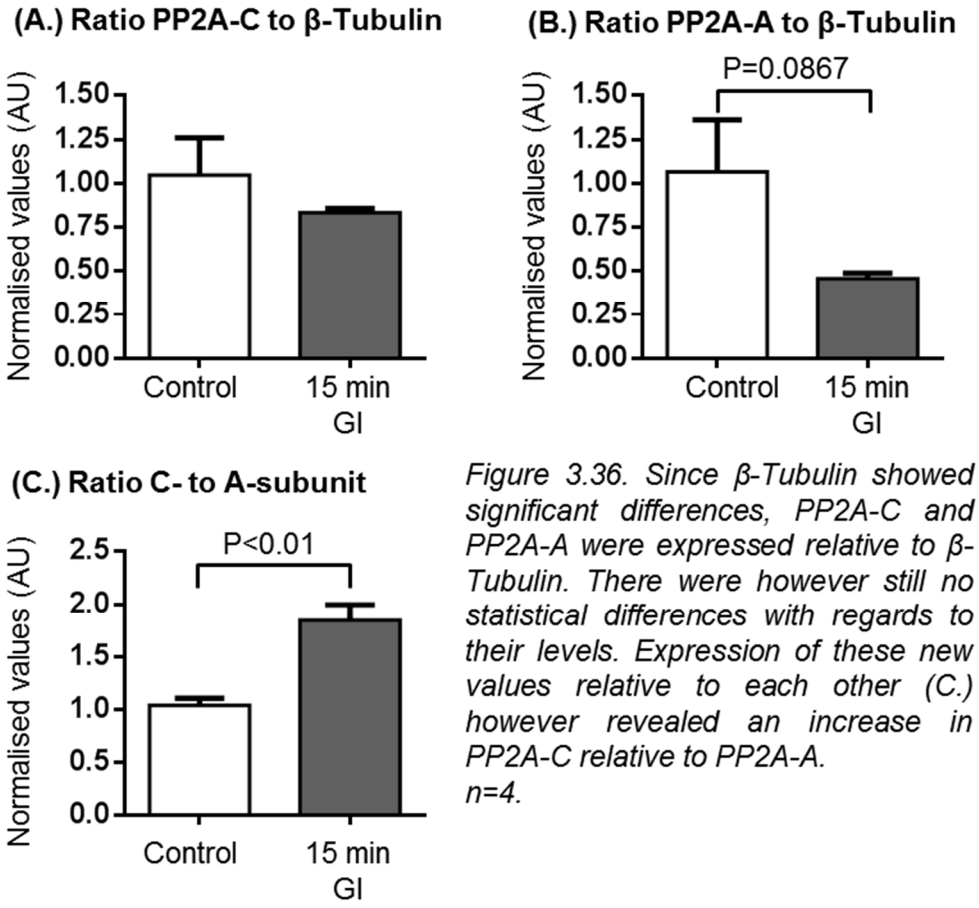


Figure 3.36. Since β-Tubulin showed significant differences, PP2A-C and PP2A-A were expressed relative to β-Tubulin. There were however still no statistical differences with regards to their levels. Expression of these new values relative to each other (C.) however revealed an increase in PP2A-C relative to PP2A-A.
n=4.

This shift in relative total protein values was not associated with any change in posttranslational modification (Figure 3.37). Adjustment of the phosphorylated and nonmethylated data for β-Tubulin and then expressing that data relative to β-Tubulin adjusted PP2A-C values did not alter the relative differences between control and 15 minutes GI, since both PP2A-C and the relevant modification is expressed relative to the same value (β-Tubulin).

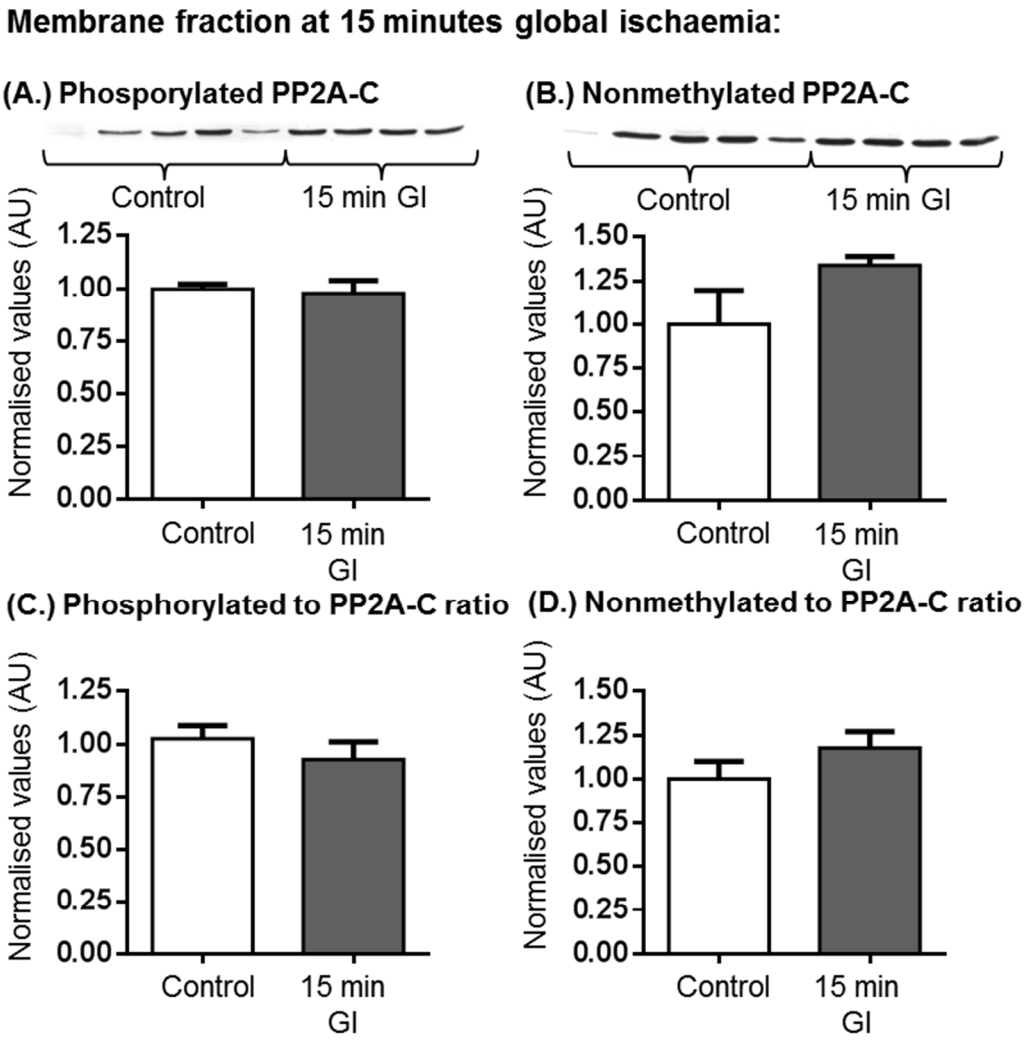


Figure 3.37. Phosphorylation and methylation of PP2A-C at 15 minutes global ischaemia (GI) in the membrane fraction. Fifteen minutes GI failed to induce any changes in either phosphorylation or nonmethylation. n=3-4.

20 minutes global ischaemia

Although 20 minutes GI did not influence total PP2A-C and PP2A-A in the nuclear fraction (Figure 3.38), it was associated with a significant increase in the value of PP2A-C relative to PP2A-A (Control: 1.00±0.08 AU vs 20 min GI: 1.38±0.13 AU; $P<0.05$). This shift was associated with a reduction in nonmethylated signal (absolute nonmethylated: Control: 1.03±0.03 AU vs 20 min GI: 0.60±0.07 AU; $P<0.01$; and relative to total PP2A-C: Control: 0.98±0.05 AU vs 20 min GI: 0.68±0.09 AU; $P<0.05$), as well as an increase in the absolute phosphorylation values (Control: 1.00±0.25 AU vs 20 min GI: 1.81±0.16 AU; $P<0.05$) (Figure 3.39).

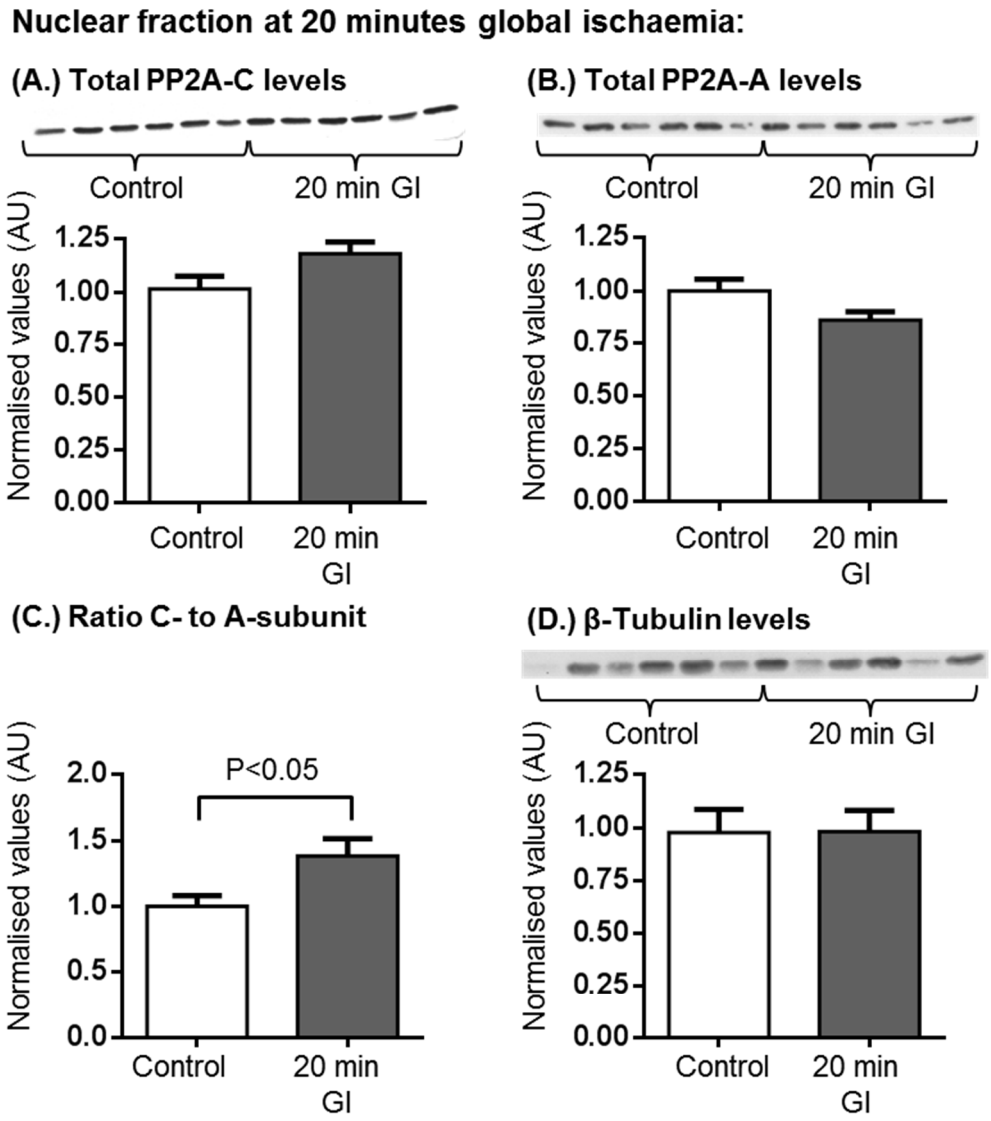


Figure 3.38. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 20 minutes GI as measured in the nuclear fraction.
Although there were no differences in the levels of PP2A-C, or –A, there was an increase in the level of PP2A-C relative to PP2A-A.
n=5-6.

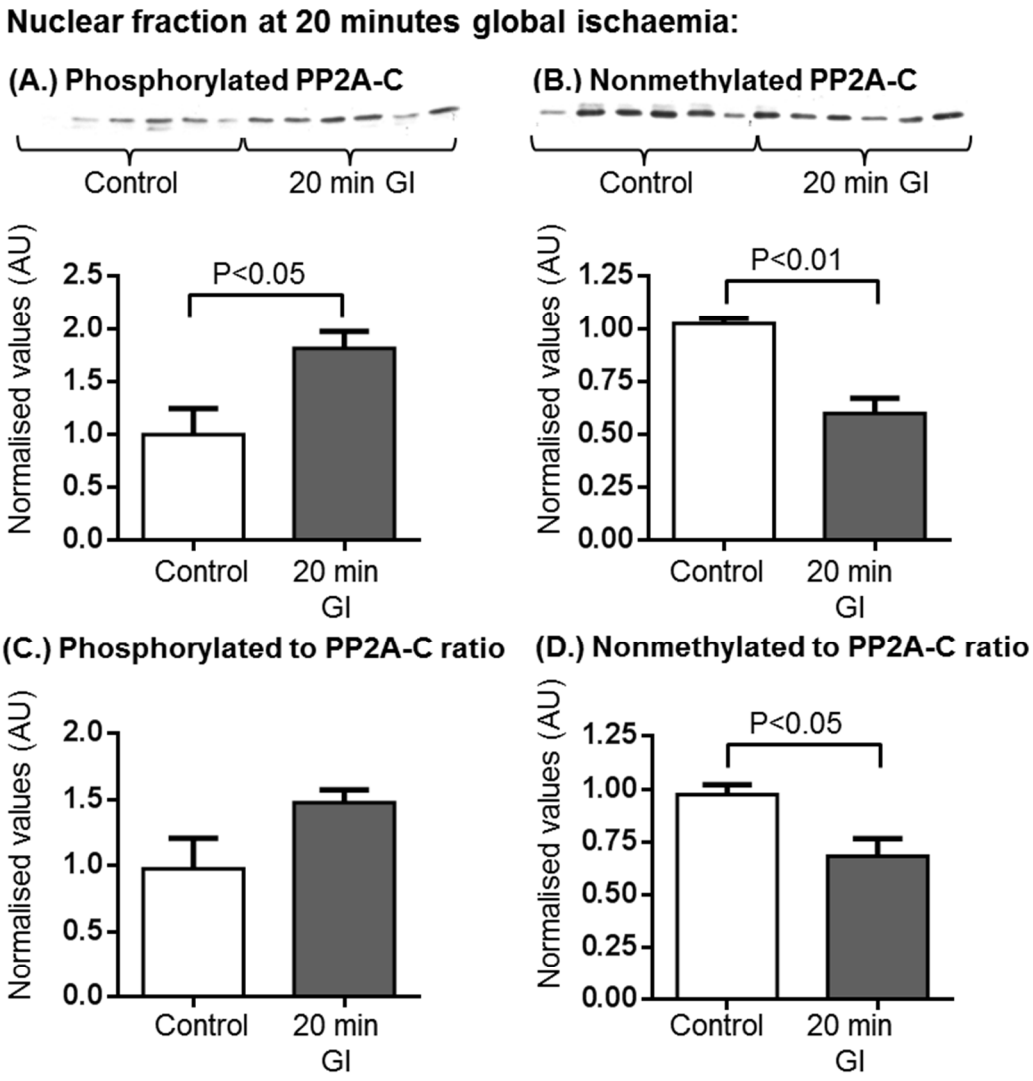


Figure 3.39. Phosphorylation and methylation of PP2A-C at 20 minutes GI in the nuclear fraction. Phosphorylation was increased at the end of ischaemia, although not relative to PP2A-C. Nonmethylation was reduced, both in absolute terms, as well as relative to total protein.
n=4-5.

In the cytosolic fraction, similarly to the nuclear fraction, there was an increase in the PP2A-C/A ratio (Control: 1.00±0.04 AU vs 20 min GI: 1.35±0.13 AU; P<0.05) in the absence of changes in either PP2A-C or PP2A-A alone (Figure 3.40). In contrast to the nuclear fraction though, this occurred in the absence of any posttranslational modifications (Figure 3.41).

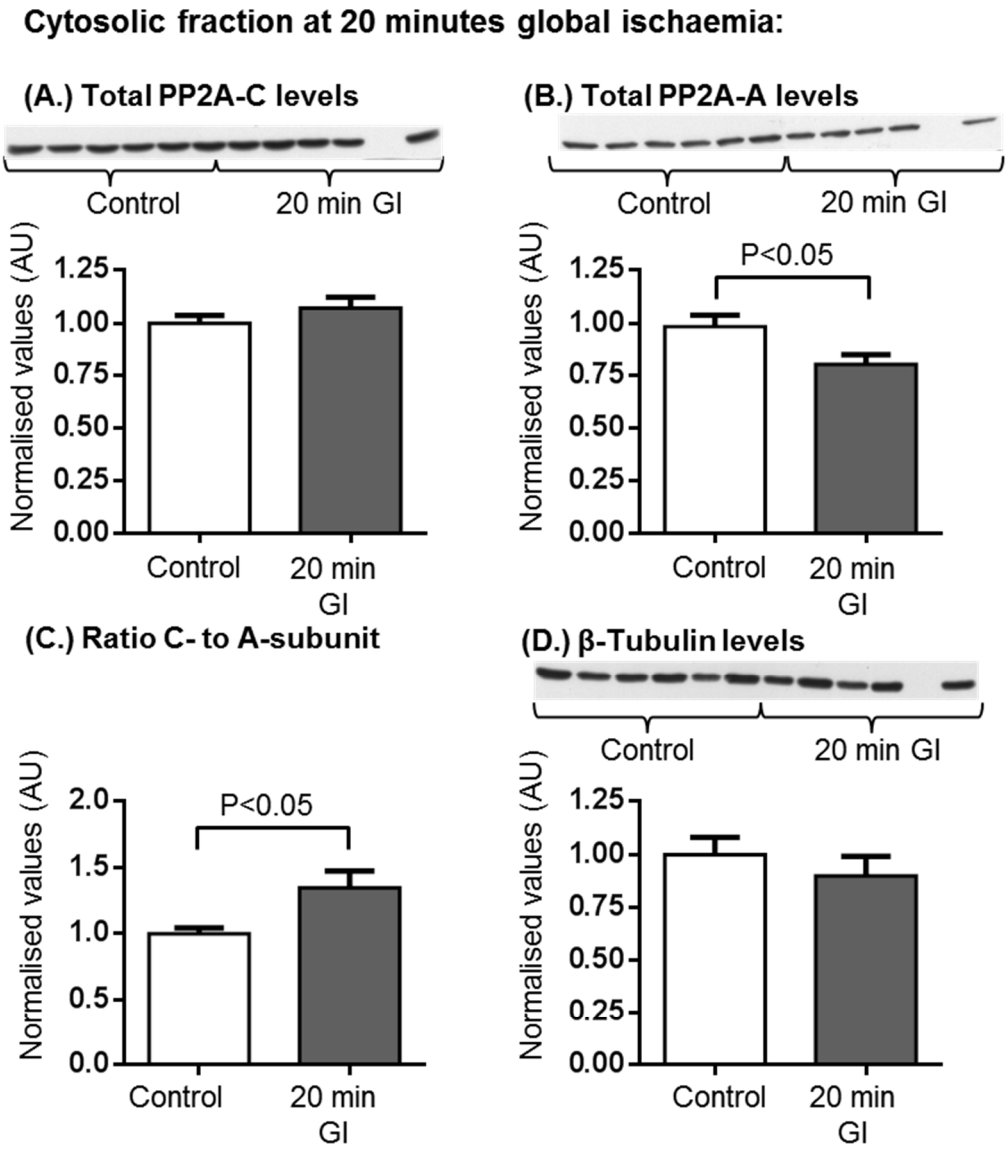


Figure 3.40. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 20 minutes global ichaemia (GI) as measured in the cytosolic fraction. This period of ischaemia reduced PP2A-A, thereby shifting the balance between PP2A-C and - A.
n=4-6.

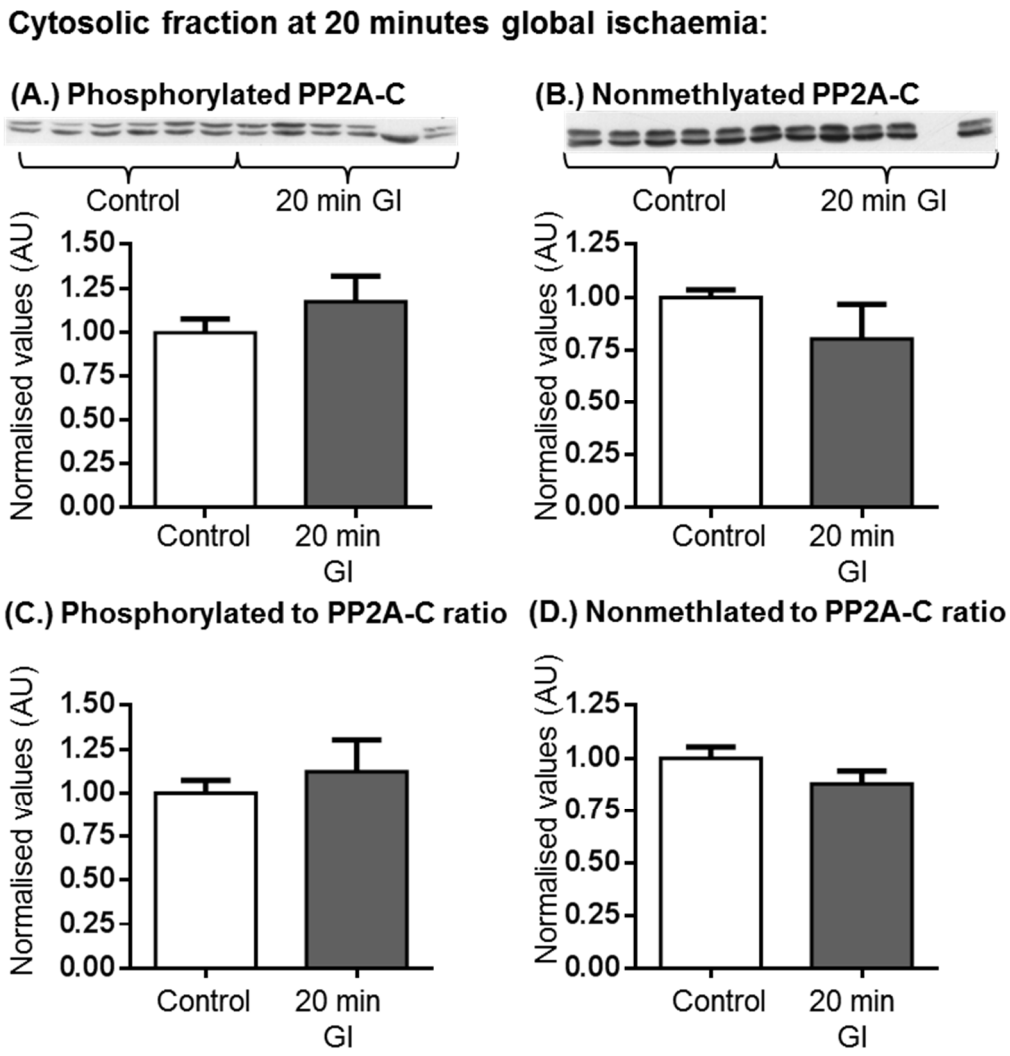


Figure 3.41. Phosphorylation and methylation of PP2A-C at 20 minutes global ischaemia (GI) in the cytosolic fraction. There were no differences with regards to any of these modifications.
n=4-6.

There were no changes in the membrane fraction (Figures 3.42 and 3.43), except for a reduction in the nonmethylated signal (absolute nonmethylated: Control: 1.00±0.06 AU vs 20 min GI: 0.60±0.06 AU; $P<0.01$; and relative to total PP2A-C: Control: 1.03±0.10 AU vs 20 min GI: 0.78±0.04 AU; $P<0.05$).

Membrane fraction at 20 minutes global ischaemia:

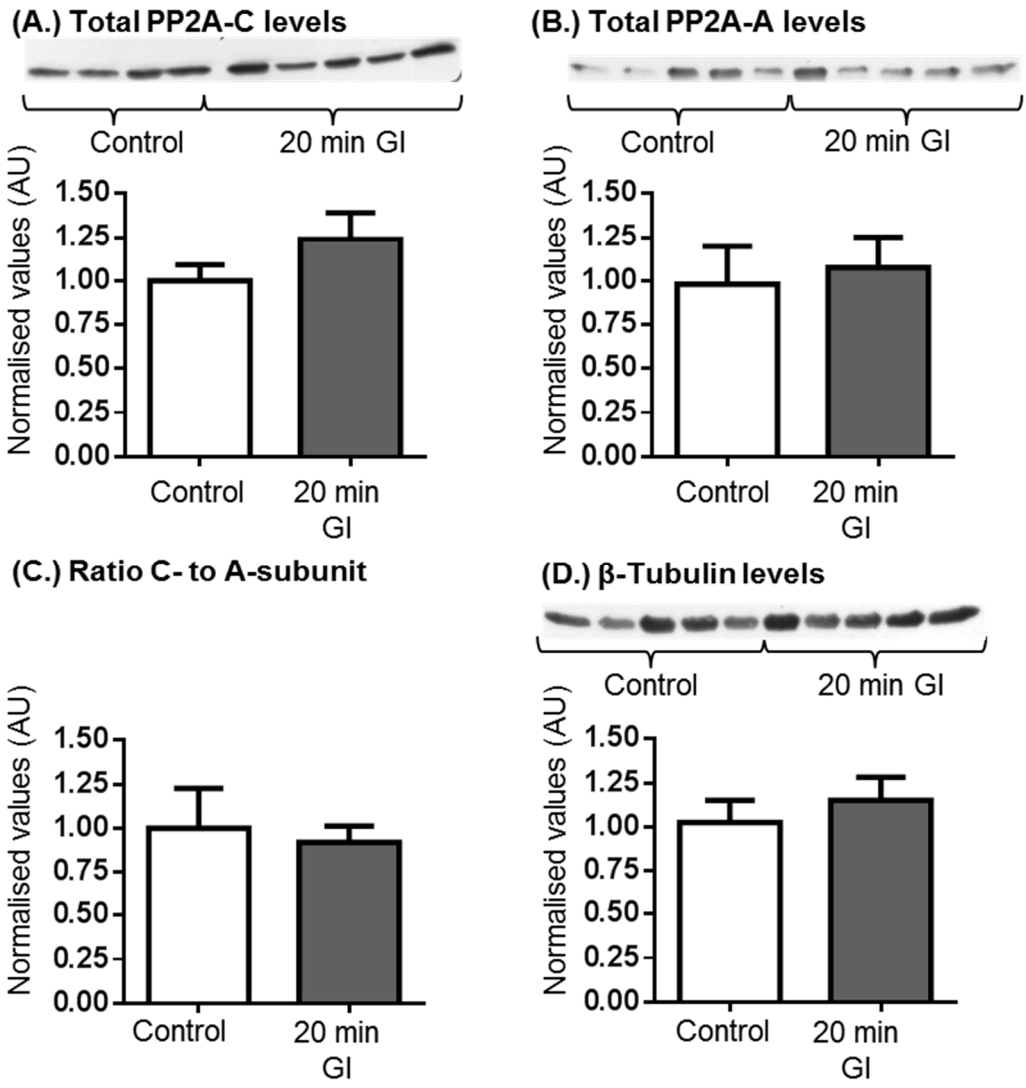


Figure 3.42. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) at 20 minutes global ichaemia (GI) as measured in the membrane fraction. n=4-5.

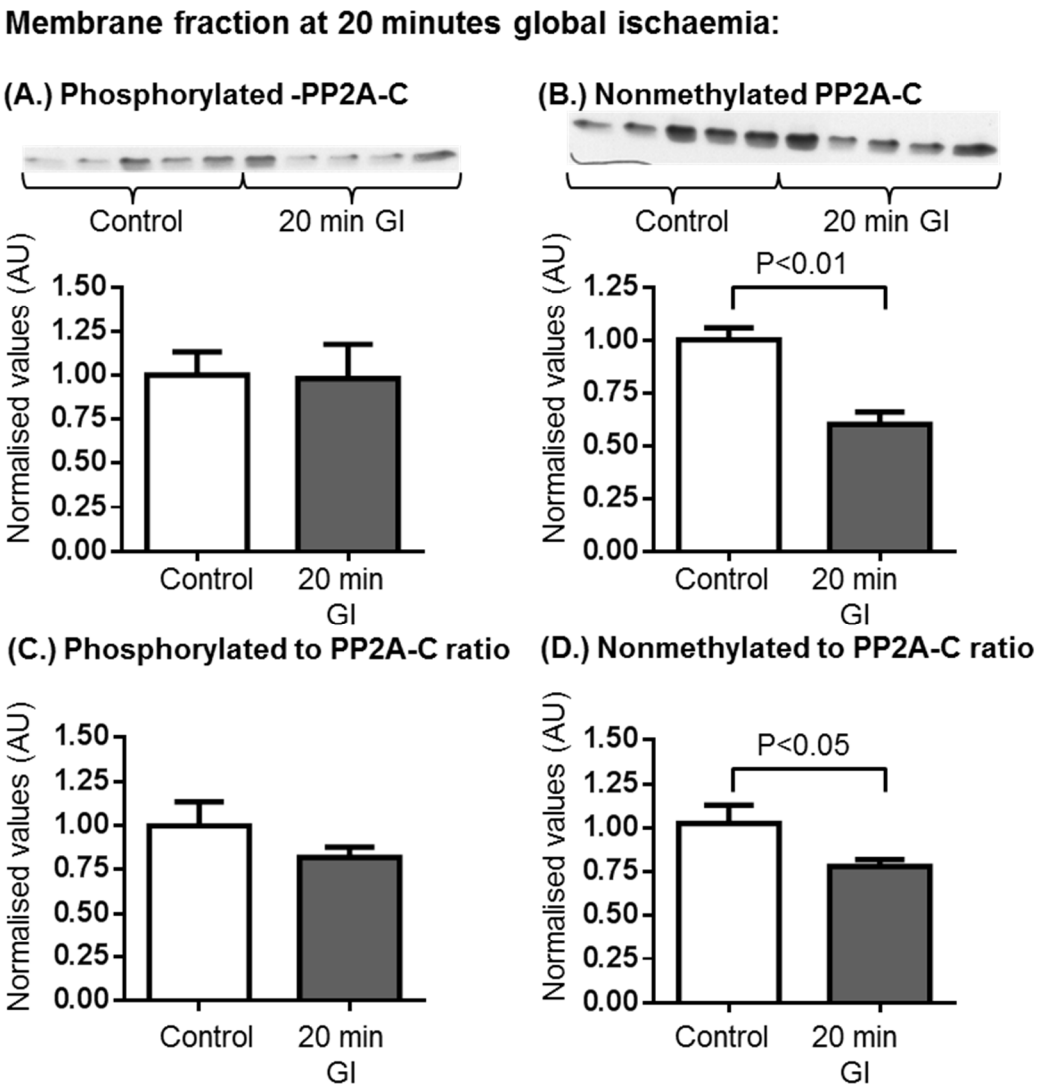


Figure 3.43. Phosphorylation and methylation of PP2A-C at 20 minutes global ischaemia (GI) in the membrane fraction. Sustained ischaemia reduced nonmethylation, both in absolute terms as well as relative to PP2A-C. n=3-5.

5 minutes reperfusion

Contrary to expectation, not many of the parameters measured changed at this timepoint (Figures 3.44, 3.47 and 3.48). There was an increase in the phosphorylation of PP2A-C in the nuclear (Figure 3.45, phosphorylation relative to PP2A-C: Control: 1.00±0.09 AU vs 5 min Repf: 1.37±0.07 AU; P<0.05), as well as the membrane (Figure 3.49, phosphorylation relative to PP2A-C: Control: 1.00±0.05 AU vs 5 min Repf: 1.41±0.08 AU; P<0.01) fractions. This indicates a probable reduction in PP2A activity in these two compartments.

Interestingly, the increase in PP2A-C/A observed in the cytosolic fraction at 20 minutes GI was maintained at 5 minutes reperfusion (Figure 3.46, Control: 1.00±0.03 AU vs 5 min Repf: 1.12±0.02 AU; P<0.01).

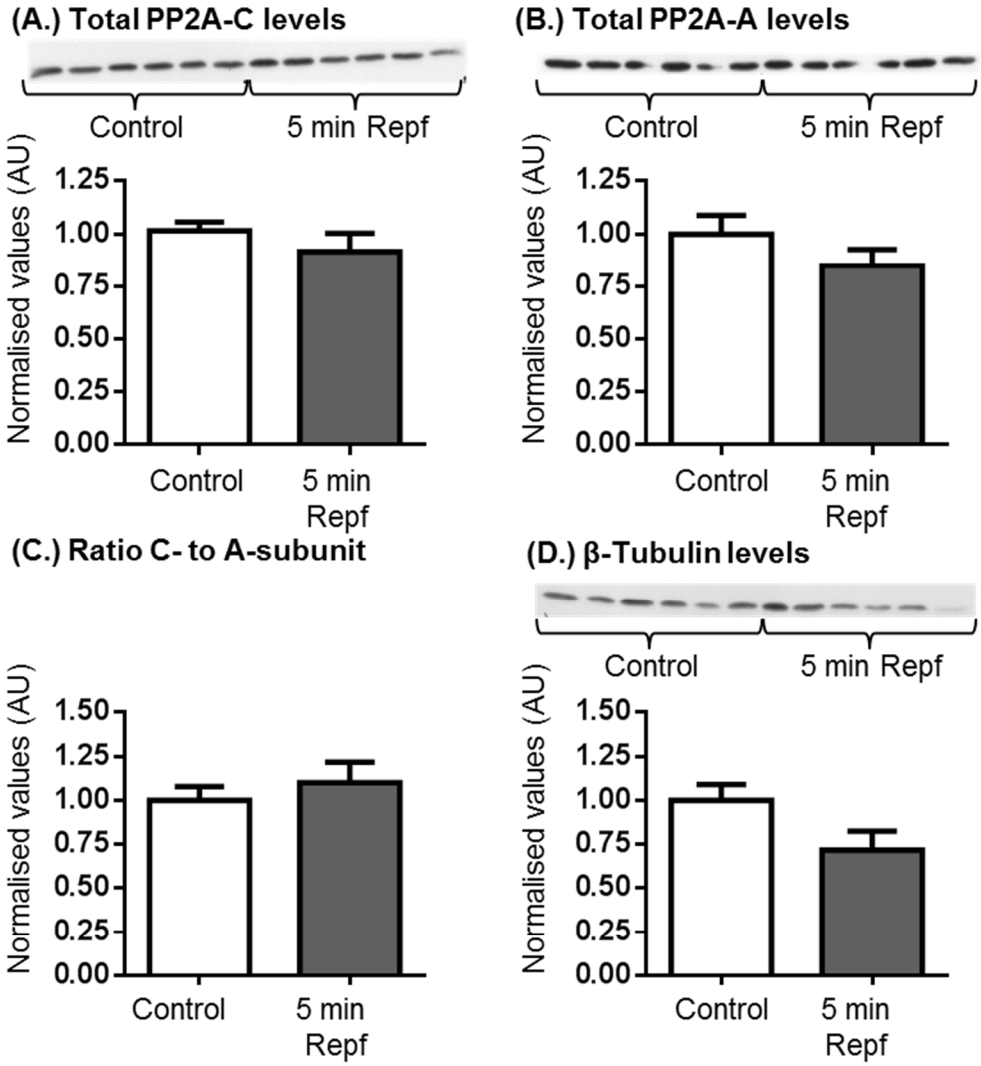


Figure 3.44. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) after 5 minutes reperfusion (Repf) as measured in the nuclear fraction.
Five minutes of reperfusion was not associated with any changes in total PP2A-C, or –A.
n=5-6.

Nuclear fraction at 5 minutes reperfusion:

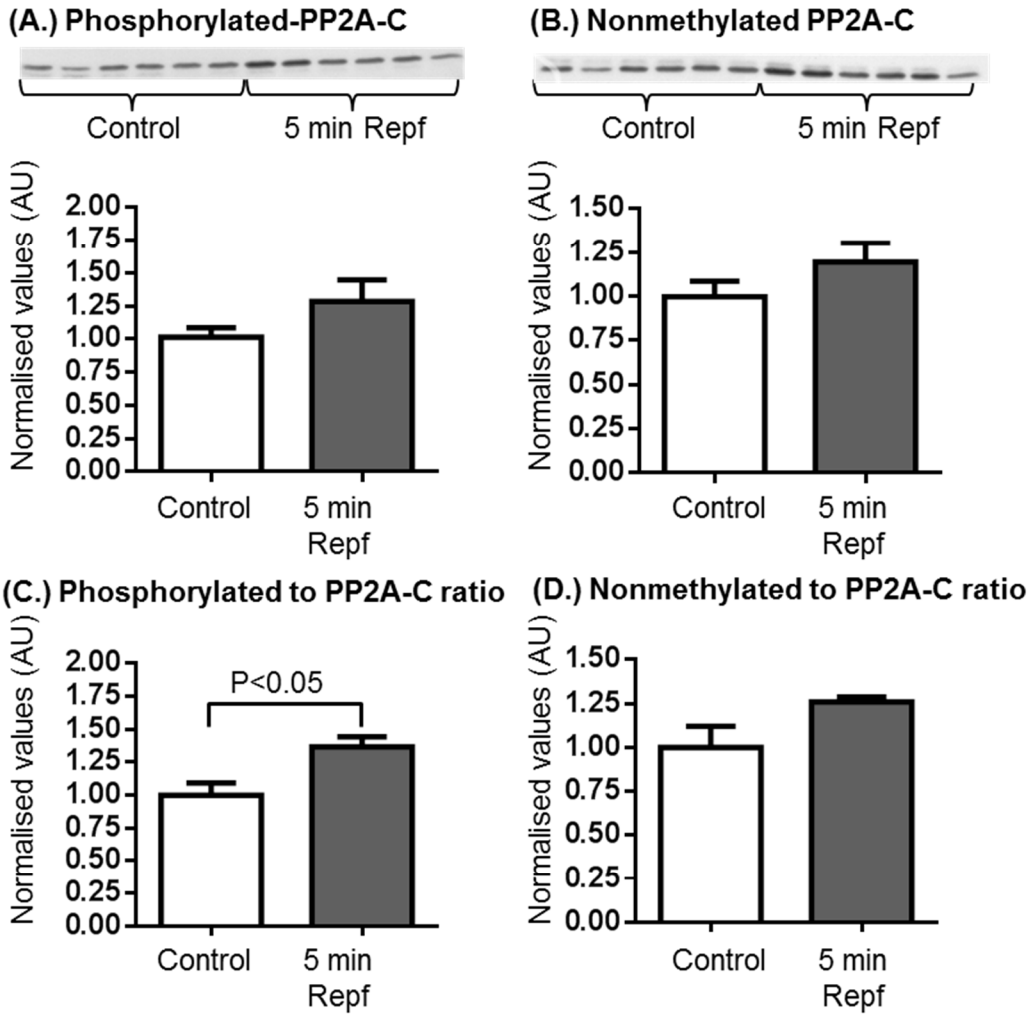


Figure 3.45. Phosphorylation and methylation of PP2A-C at 5 minutes reperfusion (Repf) in the nuclear fraction. At this time point the relative degree of PP2A-C phosphorylation was increased above control levels.
n=6.

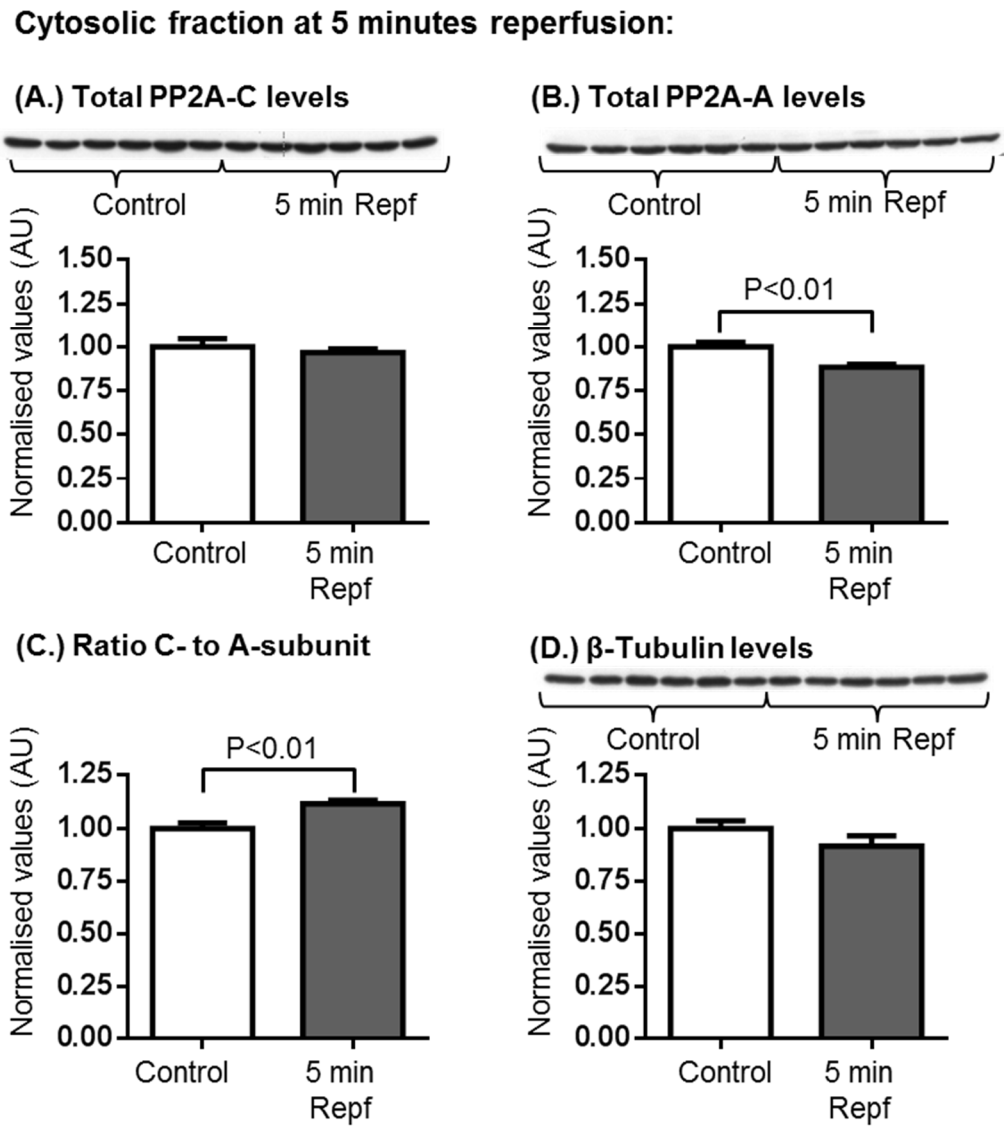


Figure 3.46. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) after 5 minutues reperfusion (Repf) as measured in the cytosolic fraction. This period of reperfusion was associated with a reduced PP2A-A, with a concomitant shift in the balance between PP2A-C and - A.
n=6.

Cytosolic fraction at 5 minutes reperfusion:

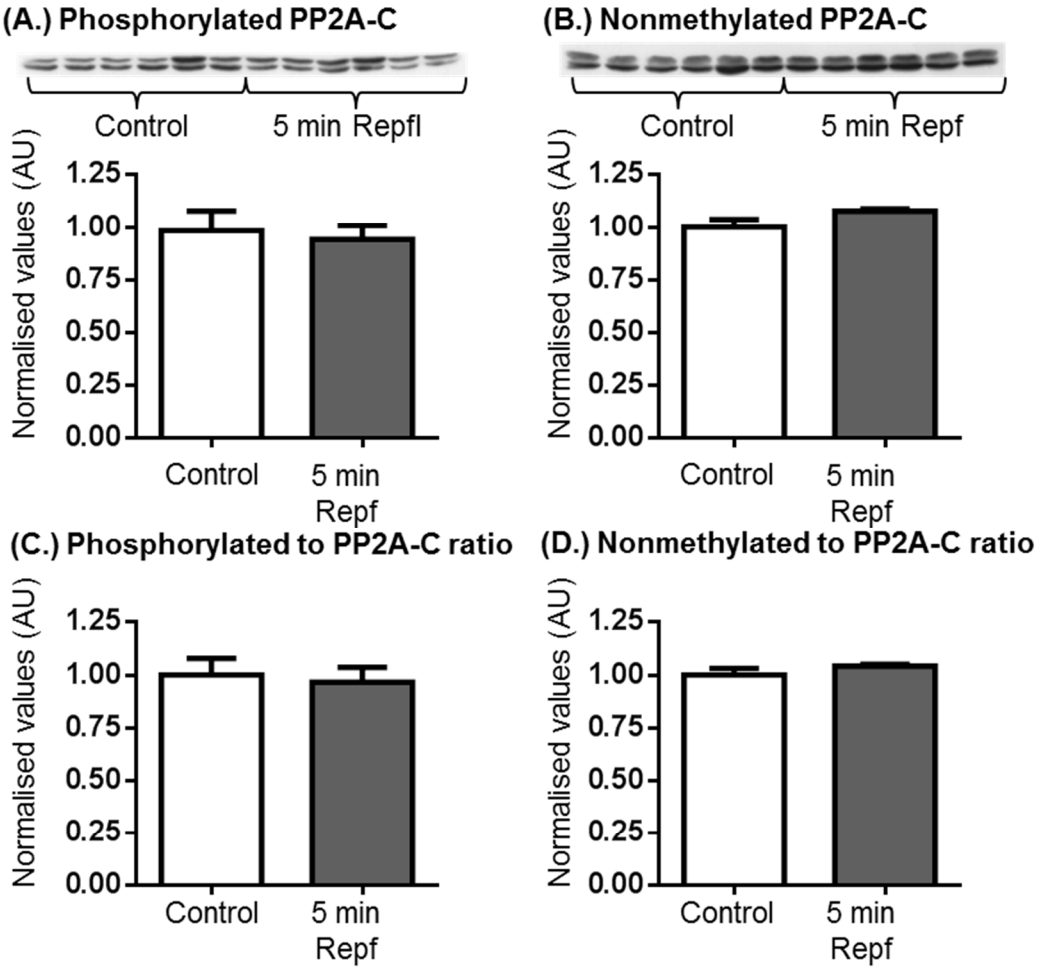


Figure 3.47. Phosphorylation and methylation of PP2A-C at 5 minutes reperfusion (Repf) in the cytosolic fraction. There were no differences with regards to any of these modifications.
n=5-6.

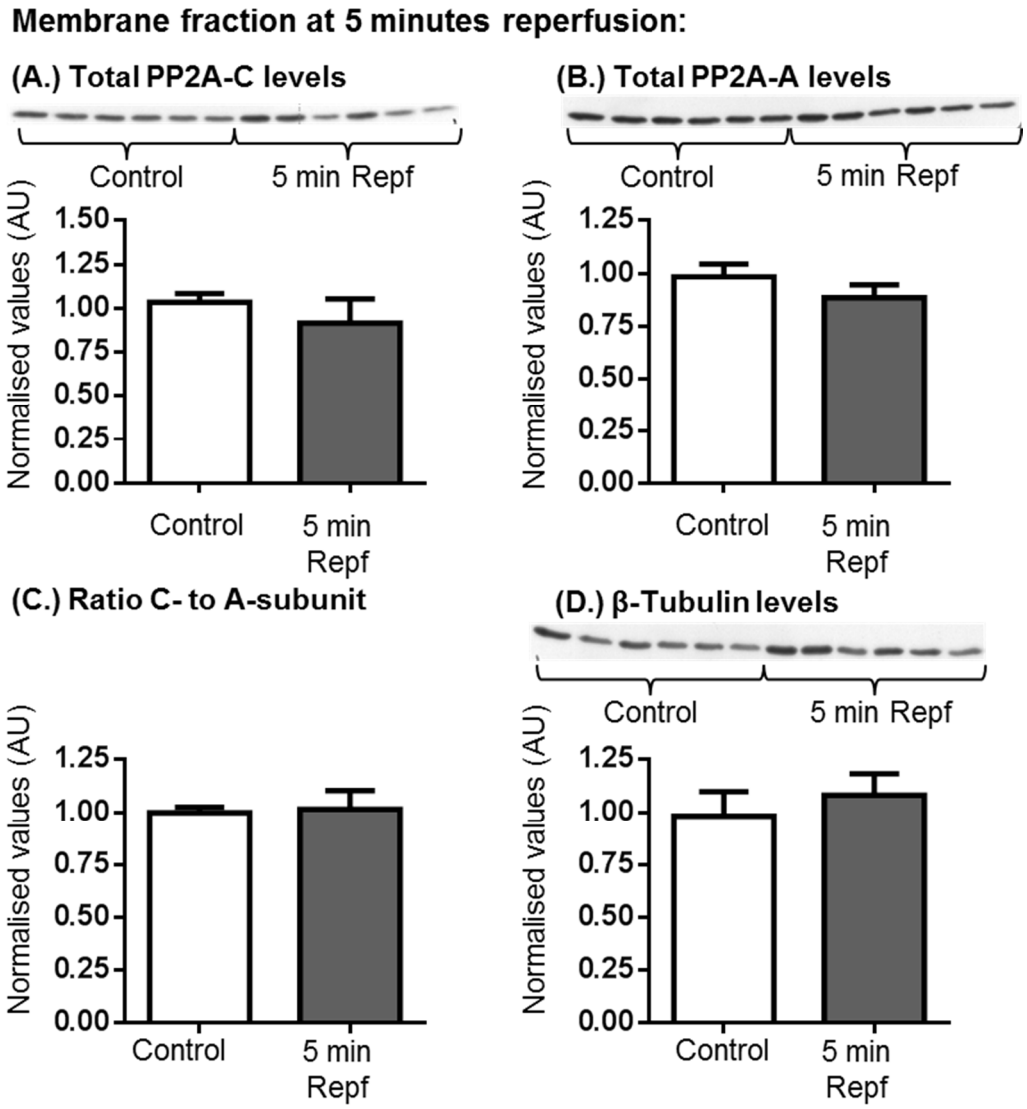


Figure 3.48. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) after 5 minutes reperfusion (Repf) as measured in the membrane fraction. n=6.

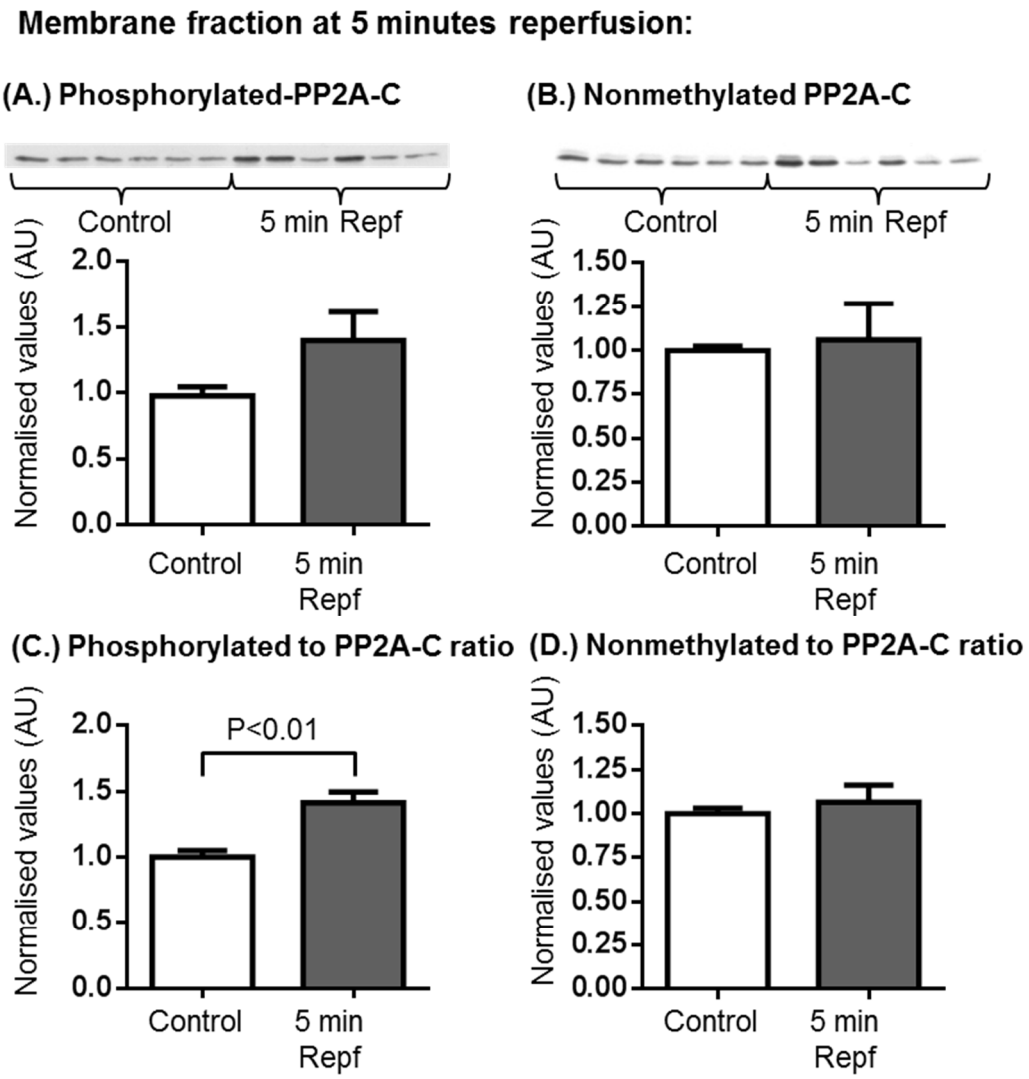


Figure 3.49. Phosphorylation and methylation of PP2A-C at 5 minutes reperfusion (Repf) in the membrane fraction. Early reperfusion was associated with an increase in the degree of phosphorylation of PP2A-C. n=5-6.

10 minutes reperfusion

Even at 10 minutes reperfusion the shift in the levels of PP2A-C relative to PP2A-A was still evident in the cytosolic fraction (Figure 3.52; Control: 1.00±0.03 AU vs 10 min Repf: 1.17±0.05 AU; $P<0.05$), in the absence of any posttranslational modifications (Figure 3.53). The observed increase in phosphorylation in the nuclear fraction at 5 minutes reperfusion was however dissipated at 10 minutes reperfusion, indeed there were no significant alterations concerning any of the measured parameters in the nuclear fraction at this time (Figures 3.50 and 3.51).

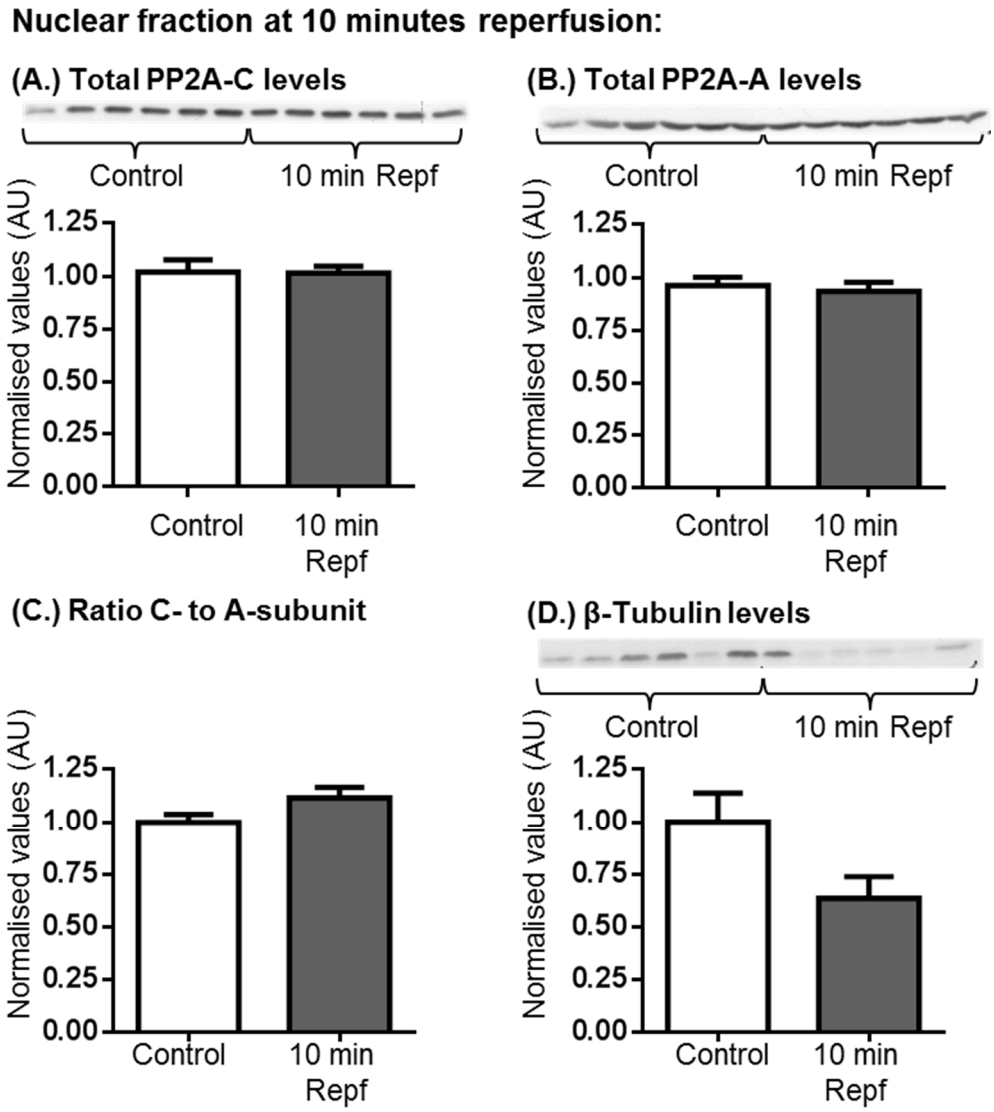


Figure 3.50. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) after 10 minutes reperfusion (Repf) in the nuclear fraction. n=5-6.

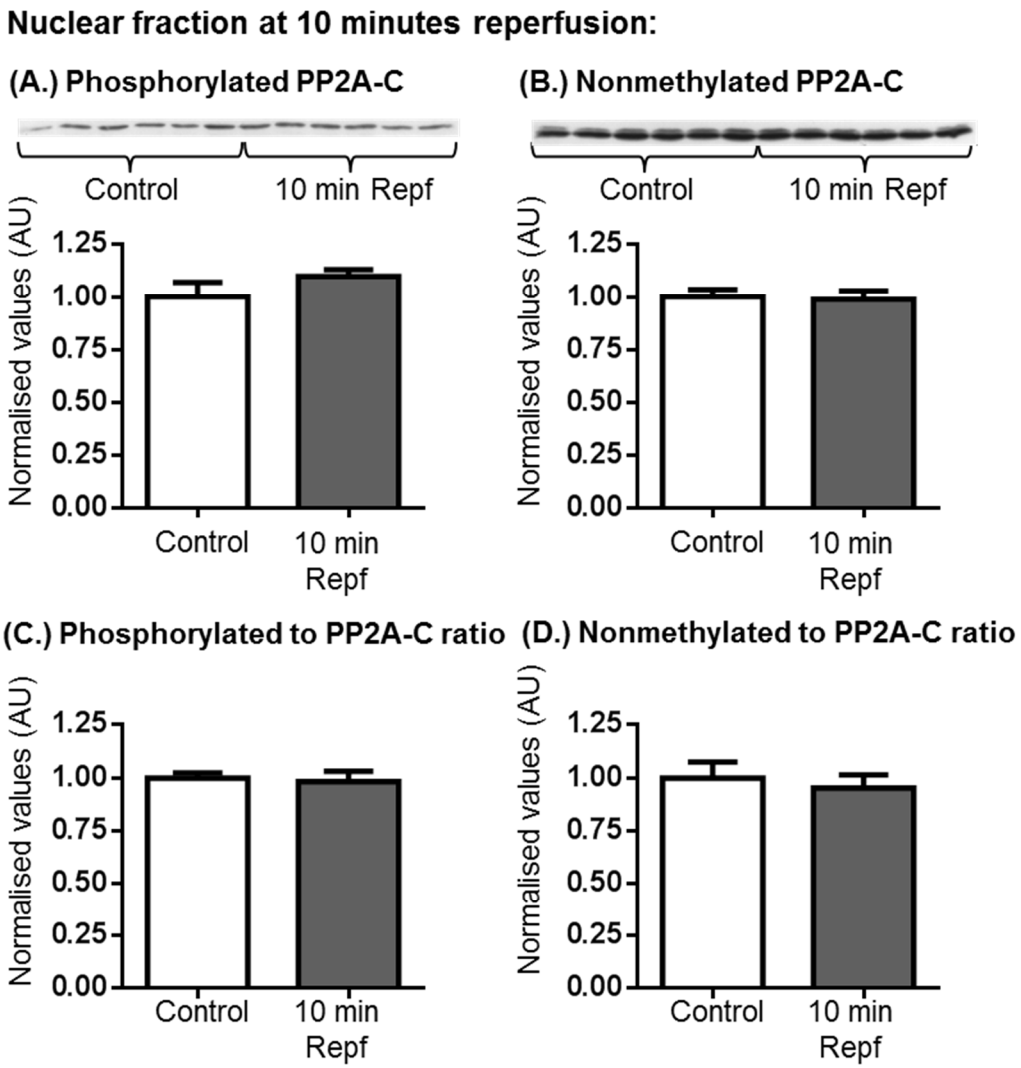


Figure 3.51. Phosphorylation and methylation of PP2A-C at 10 minutes reperfusion (Repf) in the nuclear fraction.
n=4-6.

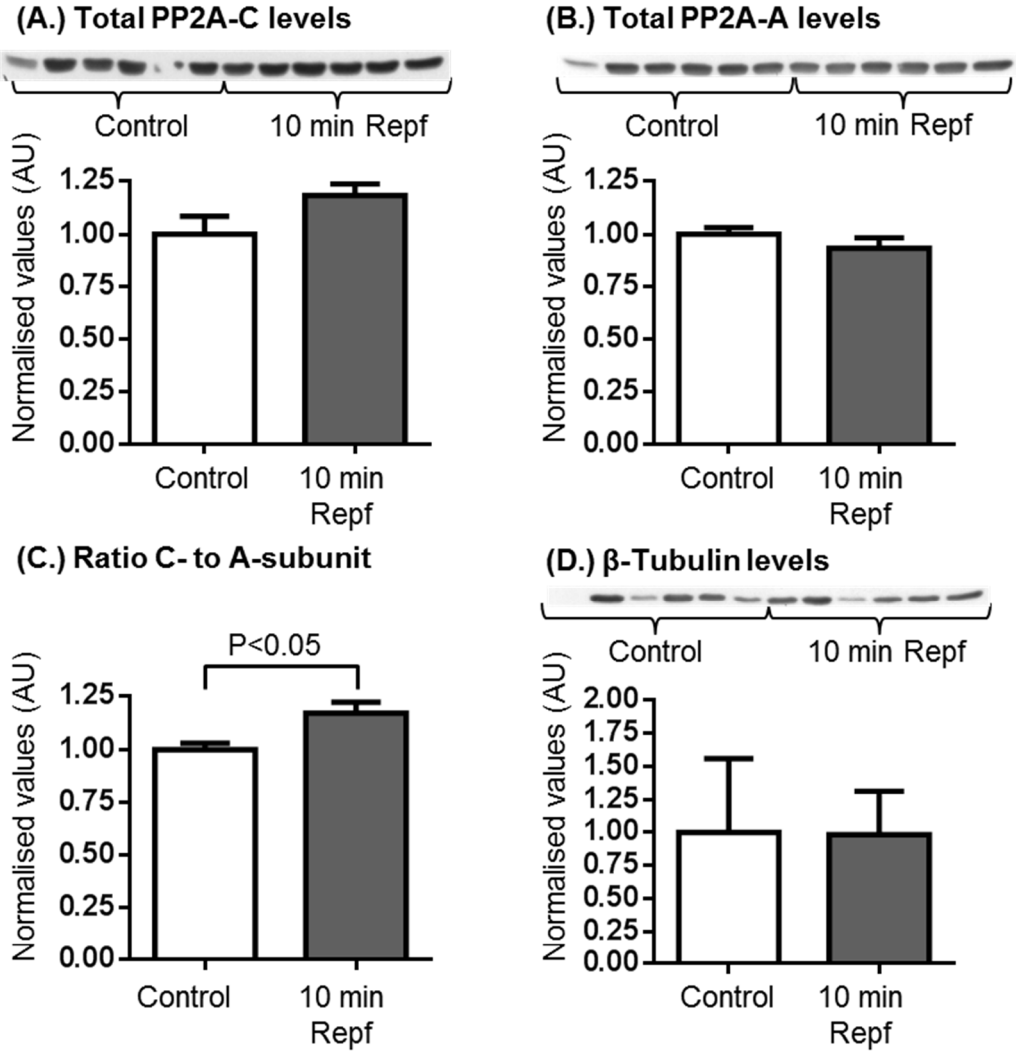


Figure 3.52. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) after 10 minutes reperfusion (Repf) as measured in the cytosolic fraction. There were no changes in the levels of either PP2A-C, or –A. Despite this, there was a slight, yet signifcant increase in the amount of PP2A-C relative to PP2A-A.
n=5-6.

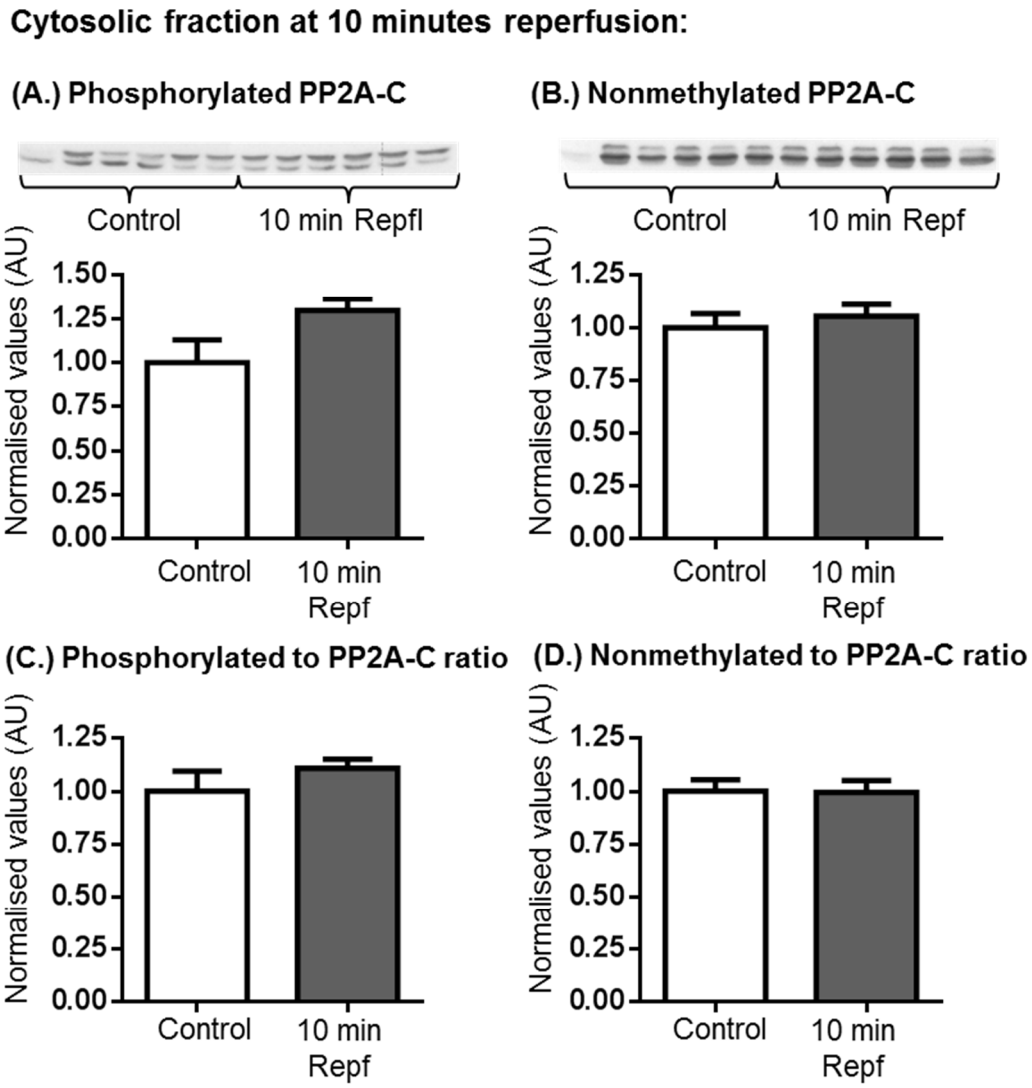


Figure 3.53. Phosphorylation and methylation of PP2A-C at 10 minutes reperfusion (Repf) in the cytosolic fraction.
n=5-6.

The methylation pattern in the membrane fraction had switched around from a reduced nonmethylation signal at the end of ischaemia, to an increase in nonmethylation at 10 minutes reperfusion (Figure 3.55, Control: 1.00±0.13 AU vs 10 min Repf: 1.5±0.14 AU; $P<0.05$). This change was not associated with any changes in total protein or PP2A-C/A (Figure 3.54).

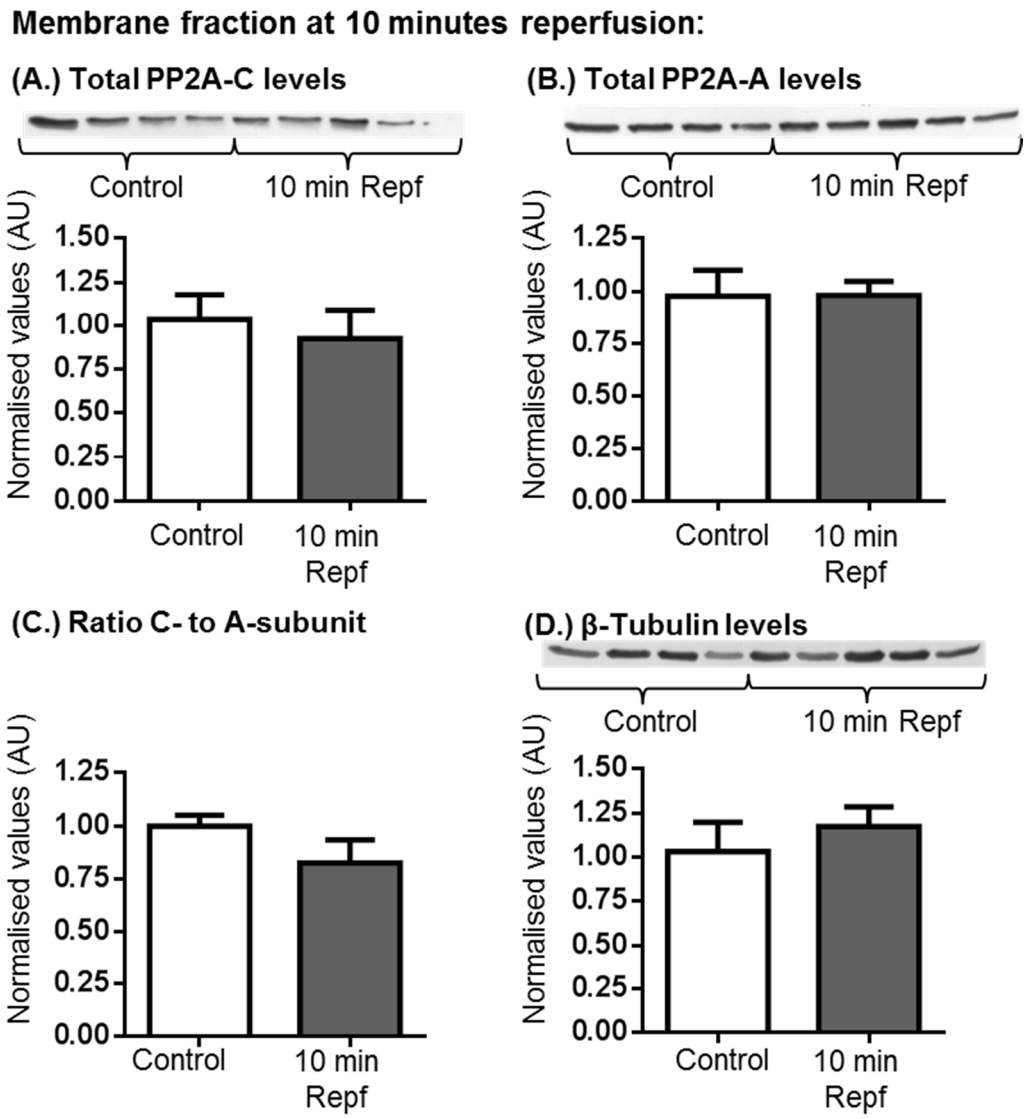


Figure 3.54. Levels of total PP2A-C (A), PP2A-A (B) and β-Tubulin (D) after 10 minutes reperfusion (Repf) as measured in the membrane fraction. No signifcant changes were observed.
n=3-5.

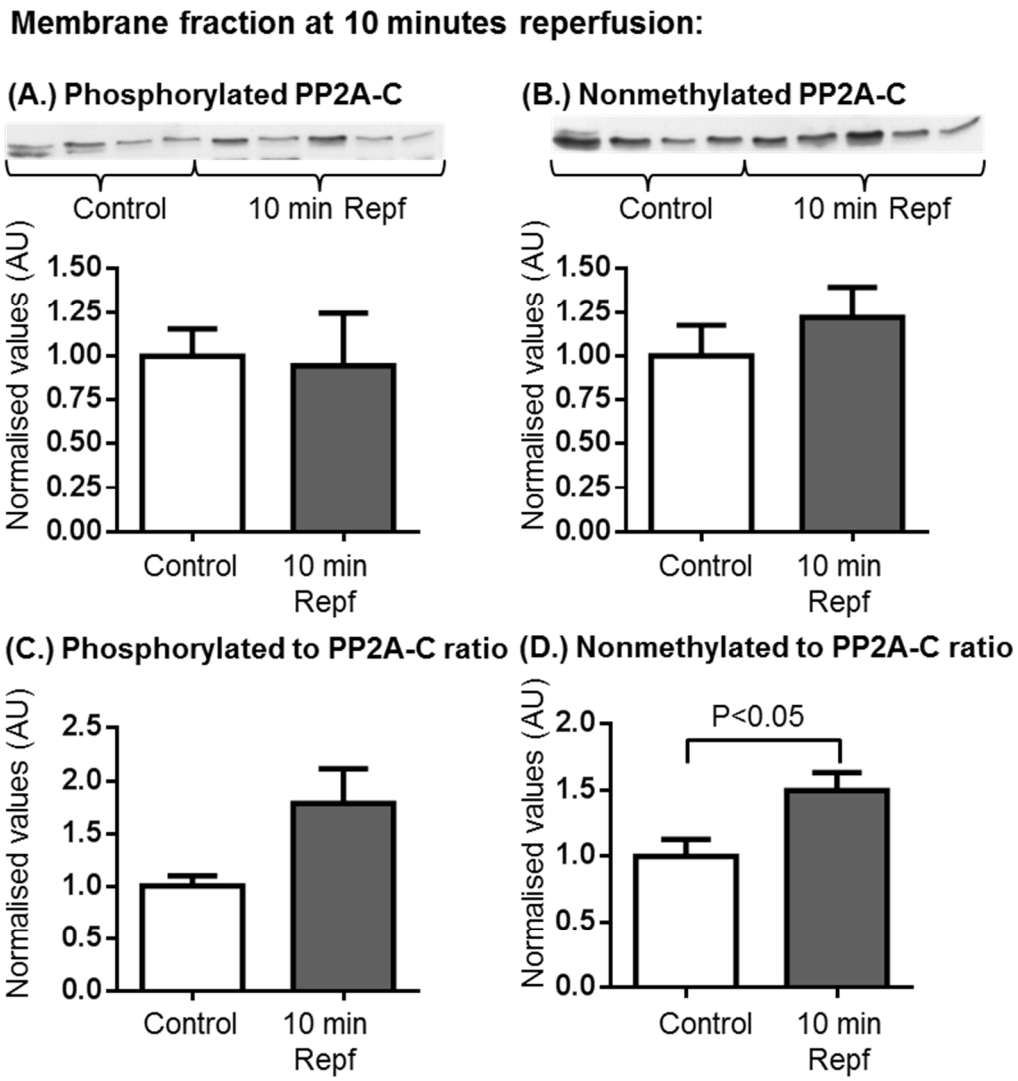


Figure 3.55. Phosphorylation and methylation of PP2A-C at 10 minutes reperfusion (Repf) in the membrane fraction. At this time there was a significant increase in the methylation of PP2A-C relative to total values. n=3-5.

Discussion

In this part of the study we endeavoured to describe the changes in PP2A levels, phosphorylation and methylation during the latter half of a severe ischaemic episode, as well as during the first moments of reperfusion. We decided to investigate the latter half of our experimental ischaemic exposure, since we expected to see the most outspoken response of PP2A to ischaemia within that time frame. Early reperfusion was of interest since it presents a clinically relevant period which has been associated with the genesis of reperfusion injury and as such, could be exploited as an opportunity for a cardioprotective intervention.

We used two very different models of I/R injury in our investigation: a cell-based model, as well as an isolated perfused rat heart model. The rationale behind this was to establish if a common

pattern could be observed in both models. The dynamics of these models are very different with regards to energy homeostasis and cellular proliferation.

The isolated heart has the ability to contract and perform work, which implies a high metabolic demand, while the cell model we used was a myoblast model of cardiac origin which does not contract or beat and therefore has a much lower metabolic demand. These differences in energy demand were especially evident with regards to the duration of ischaemia which was necessary to inflict damage: 20 minutes GI and 35 minutes RI for the isolated heart preparation versus 2 hours SI in the cell model.

The continual proliferation of the H9c2 cells could also be a confounding factor in our project, since PP2A has been found to be involved in the regulation of the transition into and out of mitosis. A detailed discussion of the participation of PP2A in the cell cycle falls outside the scope of this thesis, but in brief: It has been observed as early as 1989 that the addition of OA to proliferating cells can lead to the premature initiation of mitosis (Picard *et al.*, 1989), implicating PP1 and PP2A in the regulation of the transition into mitosis. Since then it has become clear that the transition into and out of mitosis is dependent on the activation of several kinases, of which cyclin-dependent protein kinase (Cdk1) is one of the most important (Hunt, 2013). This dependence on phosphorylation events, just as with intracellular signalling as dealt with in this text, implies the presence of phosphatase activity. Although several phosphatases are possibly involved, PP2A has emerged as a negative regulator of the transition into mitosis and has also been implicated in mediating the departure from mitosis (Jansens & Goris, 2001; Kurimchak & Graña, 2012; Hunt, 2013). This participation in the regulation of the cell cycle is therefore a role fulfilled by PP2A in the H9c2 cell line, but not in adult heart tissue, and as such poses a potential complicating factor in the comparison of our H9c2 and isolated heart results. However, for these experiments, we only exposed near confluent H9c2 cells to a relatively brief SI intervention. Taken together, these two precautionary measures should have minimized the effects of the cell cycle *per se* on our results. Any possible remaining confounding effect that the cell cycle might still have was controlled for by the control group. We therefore believe that cell cycle progression exerted a nominal effect on our results.

Determination of protein levels and posttranslational modification (i.e. phosphorylation and methylation) was done using standard Western Blotting techniques. Posttranslational modification was expressed both in absolute terms, as well as relative to total PP2A-C levels – since we considered both these values as informative. Use of an antibody specific for phosphorylated PP2A-C indicates changes in the actual levels of phosphorylation (the absolute values), and if the principles of Western Blotting hold (i.e. immunology-mediated recognition of a specific protein or modified protein), we must accept this. Expressing it as a ratio to PP2A-C gives us an additional

vantage point: it allows us to 'correct' for any possible changes in PP2A-C and it reveals the relative degree of phosphorylation of the intracellular pool of PP2A-C. The dissociation between these two values with which we are sometimes confronted (for example a reduction in absolute phosphorylation with no change in the relative degree of phosphorylation) is problematic, but could be due to small fluctuations in the intracellular content of PP2A-C or due to the lack of precision associated with Western Blotting.

Although the use of immunological techniques to investigate proteins is a powerful approach which allows for a high degree of sensitivity, laboratory experience has shown that Western Blotting lacks precision (i.e. repeatability). Possibly its high sensitivity makes it more prone to confounding influences during the experimentation process. In this sense Western blotting can be likened to using an ultrasensitive scale on a rickety old wooden bench in the middle of an undergraduate laboratory. This must be kept in mind when interpreting results, as it implies that we are looking for trends and not absolutely rigid data. Irrespective the reason, the lack of an observed change in phosphorylation relative to total levels, is indicative of a relatively small change which lacks robustness – yet it hints at a change in phosphorylation which cannot be ignored. I will follow this rationale to interpret Western Blotting data throughout this thesis.

We hypothesized that as ischaemia progresses PP2A activity will remain stable, or possibly increase during the later periods of ischaemia in association with a global cellular reduction in ATP levels. Reperfusion would however reverse this by inducing a reduction in PP2A activity. Since methylation of PP2A-C is closely linked to its substrate specificity and subcellular distribution, we also expected to see changes in methylation as ischaemia progresses followed by a reversion back to pre-ischaemic patterns during reperfusion, although the timeframe for this possible return to baseline is unknown. Previous work done in our laboratory had found that kinase phosphorylation had returned to control levels at 30 minutes reperfusion in the isolated rat heart exposed to 20-35 minutes ischaemia. It is therefore very probable that PP2A would also return to its control-levels and distribution at this time.

PP2A in unfractionated "complete cell" preparations

Data from the H9c2 cell model

The most interesting observation made in this model of ischaemia was the transient reduction in total PP2A-A levels at 60 minutes of SI (Figure 3.6) and again at 15 minutes reperfusion following 120 minutes SI (Figure 3.12). This suggests that in the relatively early stages of a stress situation, either ischaemia or reperfusion, a shift in the balance between the synthesis and degradation of PP2A-A occurs. A reduction in PP2A means that either the rate of synthesis was inhibited, or the rate of degradation was increased. PP2A-A is a relatively stable protein with a half-life of

approximately 10 hours (Zhou *et al.*, 2003). This being said, there was an increase in the levels of PP2A-C relative to PP2A-A at 60 minutes SI (Figure 3.6), which was also associated with a reduction in the methylation of PP2A-C. Taken together this could indicate a dissociation of the PP2A dimer or trimer, with PP2A-C being demethylated and stabilised in association with another molecule – possibly α4, which preferentially binds to nonmethylated PP2A-C thereby stabilizing and inactivating it (Chung *et al.*, 1999; Kong *et al.,* 2009; McConnel *et al.*, 2010). Since assembly into the dimer or trimer stabilizes the different components of PP2A (Silverstein *et al.*, 2002), the dissociated PP2A-A would then become unstable and be broken down - as has been shown to happen with PP2A-C, B and B' in the absence of PP2A-A (Strack *et al.*, 2004).

Intriguingly a similar pattern was seen at 120 minutes SI: an increase in PP2A-C/A in association with a reduction in non-methylated PP2A-C levels (Figures 3.10 & 3.11). Although these shifts are statistically significant, their magnitude and physiological importance may be doubtful, since these changes were not observed at 90 minutes SI and the PP2A-C/A shift at 120 minutes SI was not accompanied by a reduction in PP2A-A nor was the nonmethylation signal statistically elevated when expressed relative to PP2A-C. Inversely, the reduction in PP2A-A at 15 minutes reperfusion did not lead to a change in PP2A-C/A at that time, nor was it associated with a change in methylation status.

If the above theory is however true, then the observed reduction in PP2A-A is due to an increase in degradation, rather than reduced synthesis.

The absolute levels of PP2A-C phosphorylation was reduced at 15 minutes reperfusion (Figure 3.13), indicating a surprising increase in PP2A activity in early reperfusion. Although the ratio of phosphorylated PP2A-C to total PP2A-C did not change at 15 minutes, the observation of an increase in phosphorylation must not be ignored, since it indicates a slight increase in PP2A activity.

At 30 minutes reperfusion though, all the parameters investigated seem to have returned back to pre-ischaemic (i.e. control) patterns (Figures 3.14 and 3.15). This means that in this model of I/R injury PP2A does not participate in the more long term consequences of I/R.

Conclusion

In the H9c2 cardiomyoblast cell line PP2A is already implicated in its stress response at 60 minutes simulated ischaemia. After 30 minutes of reperfusion, following 2 hours of SI, PP2A levels and its posttranslational modifications return to control values. There are four noteworthy results / trends that we see in these experiments: (1.) the stability of PP2A-A levels are reduced during both ischaemia and reperfusion; (2.) The ratio of PP2A-C to PP2A-A levels increases during ischaemia;

(3.) the methylation of PP2-C is reduced during ischaemia; and (4.) phosphorylation of PP2A is reduced in early reperfusion. Taken together our results implicate the involvement of PP2A, especially during ischaemia, with regards to changes in methylation associated with changes in PP2A enzyme assembly. This has certain, unknown, implications for the cellular distribution and substrate targeting of PP2A. As proposed, ischaemia did not influence PP2A activity, although early reperfusion was associated with a slight reduction in PP2A phosphorylation – suggesting an increase in PP2A activity.

Data from the isolated rat heart

We were very surprised by the increase we saw in PP2A-C and PP2A-A levels at 20 minutes of GI in comparison to even 10 minutes GI (Figure 3.16). We therefore decided to strengthen our statistical analysis by increasing the n-value. Throughout this project we repeated these protocols in conjunction with pharmacological based studies (Chapters 4 and 5). This worked in our favour, because it meant that we had data collected over a period of approximately 4 years and spanning 3 different experiments which we could pool, thereby strengthening our results. After normalizing all data obtained to the common control condition, subsequent analysis showed that PP2A-C levels were indeed elevated at 20 minutes GI, in contrast with PP2A-A (Figure 3.18).

In the original study this elevation was temporarily suppressed at 5 minutes reperfusion and then re-emerged at 10 minutes reperfusion – a pattern which was extremely surprising. This sudden reduction in total PP2A-C and PP2A-A could be explained by the wash-out of cellular content released from damaged and dead cells in the first minutes of reperfusion. The ensuing increase which was measured at 10 minutes reperfusion was however extraordinarily rapid. In light of these unlikely results, we therefore also followed the same rationale as for the 20 minutes GI data and pooled all our available reperfusion data from other experiments (Figures 3.21 and 3.22). This showed a much more probable normalisation of the 20 min GI elevated PP2A-C back to control levels at 5 minutes reperfusion (possibly accelerated by the washout of cellular content) which then remained stable at 10 minutes reperfusion. PP2A-A actually showed a significant, but very transient, dip in its levels at 5 minutes reperfusion. By 10 minutes reperfusion both PP2A-C and –A levels had returned to control levels and remained there at 30 minutes reperfusion.

We therefore clearly found that especially ischaemia induced changes in the total levels of PP2A-C and PP2A-A. This was surprising taking into account the rate at which it happened, with the changes reaching significance within 10-20 minutes. It has been shown that PP2A-C levels are maintained within very narrow limits by an autoregulatory mechanism (Baharians *et al.*, 1998) resisting overexpression, which make our results even more surprising. However, others have also shown that hypoxia or ischaemia is associated with an increase in cellular PP2A: Larsen *et al.* (2008) found that chronic exposure (14 days) to hypoxic conditions (10% oxygen) led to an

increase in PP2A-C mRNA levels in the left ventricles of C57Bl/6j mice; while Totzeck and colleagues (2008) showed that PP2A-A expression was increased by 154% in the hearts of minipigs exposed to 85 minutes of low flow ischaemia. Although these studies differ from our experiments with regards to animal species tested, as well as the nature of ischaemia / hypoxia applied, they both illustrate that it is conceivable for PP2A to be upregulated during exposure to ischaemia.

How is this rapid increase in protein levels accomplished? Such an elevation must be due to either an increase in the synthesis or a reduction in degradation of PP2A-C. PP2A-C has been reported to have a half-life of 13 – 16.5 h (Lizotte *et al*., 2007; Baharians *et al*., 1998) making it a very stable protein, with rates of synthesis and degradation probably being quite low. The rate at which these changes in our experiments occurred favours a protein degradation mechanism, i.e. either proteasomal degradation or autophagy, where ischaemia exerts an inhibitory effect on the degradation of PP2A-C. As reported in Chapter 1, ischaemia has been shown to be associated with an increase in the occurrence of autophagy (Hamacher-Brady *et al.*, 2006; Matsui *et* al., 2008; Nishida *et al.*, 2009). It is therefore unlikely that autophagy is involved in the modulation of PP2A-C levels which we observed. Myocardial ischaemia however does induce proteasomal dysfunction, specifically the ubiquitin proteasome system (UPS) develops a diminished ability to process ubiquitinated proteins – i.e. proteins tagged for proteolysis are not degraded (Calise & Powell, 2013). As mentioned previously, through its association with α4 and MID-1 PP2A-C can be ubiquitinated and possibly, though not necessarily, be broken down as a result (Trockenbacher *et al.*, 2001; McConnell *et al*., 2010). There is however a major flaw in this theory, namely that it seems as if the autoregulation of PP2A under normal conditions relies more on regulation of the translational processing of PP2A, than the turnover thereof (Baharians & Schöntal, 1998). This idea was later confirmed by Wei *et al*. (2006) who reported that inhibition of the proteosomal system does not influence the levels of either total PP2A-C or PP2A-A. Lizotte *et al*. (2007) however reported that 12 hours of incubation with a proteasome inhibitor led to the accumulation of especially mono-ubiquitinated PP2A-C, indicating the potential relevance of the proteosomal system in regulating PP2A-C levels. Mono-ubiquitinated PP2A-C has a molecular weight of 44 kDa, we however did not see such a band in our blots. In conclusion, it is therefore theoretically possible that the reduced UPS activity associated with ischaemia could favour an increase in PP2A levels, although the contribution of this to the significant increase in PP2A-C that we observed is unknown.

By process of elimination this leaves us then with a mechanism entailing an increase in the synthesis of PP2A-C as ischaemia progresses. The question is however if an increase in PP2A expression could occur within 20 minutes? In their study on the ability of the *ob/ob* mouse heart to be protected by postconditioning, Bouhidel and co-workers (2008) reported a PostC-mediated

significant increase in the levels of MKP-1 and PTEN only ten minutes after the application of PostC. Lizotte and co-workers (2007) found that a significant amount of PP2A-C can be synthesised within 20 minutes. These studies therefore show that in principle it is possible that an increased expression of PP2A-C could explain the elevation observed at 20 minutes GI (Figures 3.16 and 3.18) . This increase in synthesis must however be very transient, since we did not see a sustained elevation during reperfusion following 20 minutes GI (Figures 3.21, 3.22 and 3.23).

In their characterization of PP2A in the heart, DeGrande and colleagues (2013) reported that both PP2A-C and PP2A-A were elevated 5 days after myocardial infarction in a canine model. This elevation was also present in human ischaemic heart failure models. Combination of our data and DeGrande's results leads to the hypothesis that ischaemia stimulates an increase in PP2A levels, which is then sustained during the development of heart failure. Our reperfusion results however contradict this model, exposing the gap in both studies – namely sufficient temporal characterization of PP2A. The elevation we observed is an acute response to ischaemic stress and damage. Their data encompasses not only I/R injury, but also the compensatory mechanisms involved in the development of heart failure after 5 days. However, no information is available concerning what happens to PP2A in the interim period between ischaemia and ischaemic heart failure. Our data suggest a transient reduction in PP2A.

An increase in cellular PP2A-C content does not necessarily imply an increase in the activity of the enzyme. We found that at the end of sustained ischaemia there was an increase in the absolute levels of PP2A-C phosphorylation (Figures 3.17 and 3.18). This increase was however due to the accompanying increase in PP2A-C levels, as evident by the unchanged phosphorylated to total PP2A-C ratio. This means that in absolute terms there was an increase in PP2A activity as ischaemia progressed, coupled to the increase in PP2A-C levels. The onset of reperfusion however definitely inhibited PP2A activity as seen by the increase in PP2A-C phosphorylation relative to the total population of enzyme at 5 and 10 minutes reperfusion (Figures 3.20, 3.21 and 3.22). This increase was however dissipated after 30 minutes of reperfusion (Figure 3.24). In summary, our data show that as ischaemia progresses PP2A-C levels increase, in conjunction with an increase in activity (dephosphorylated enzyme), which is then inhibited by early reperfusion.

Although the original experiments showed a change in the methylation of PP2A-C relative to total PP2A-C (Figure 3.17) a much more pronounced trend was exhibited by the pooled data (Figure 3.18). Here we see that sustained ischaemia favours demethylation of PP2A-C, as determined by an antibody which recognizes nonmethylated PP2A-C. This change in methylation pattern is indicative of changes in holoenzyme assembly, subcellular distribution and substrate specificity. In relation to this it is noteworthy that this increased demethylation was associated with an increase in the PP2A-C/A ratio, indicating a possible dissociation between PP2A-C and PP2A-A. Such a

dissociation might explain the slight reduction in total PP2A-A at 5 minutes reperfusion (Figure 3.21). This same methylation pattern was observed during reperfusion, in the absence of an increased PP2A-C/A ratio at 10 minutes reperfusion. Fluctuations in methylation were however abolished at 30 minutes reperfusion.

In conclusion

Our data clearly implicates PP2A as a participant in the processes involved with I/R, since both the expression and posttranslational modification of PP2A were altered by ischaemia, as well as early reperfusion. At the end of 20 minutes GI both PP2A-C and PP2A-A levels were significantly elevated in the isolated heart model. This elevation was very specific for ischaemia, since it dissipated during reperfusion. The increase in PP2A levels was also associated with an almost matched increase in phosphorylation (i.e. reduced activity) and reduction in methylation, along with an increase in the ratio of PP2A-C to PP2A-A. Early reperfusion was also characterized by an increase in phosphorylation which implies a reduction in PP2A activity.

Having summarized our results from both the H9c2 cells exposed to SI and the isolated rat heart undergoing 20 minutes GI, the next section will focus on the combination of these two.

What does the combination of the H9c2 cell and isolated rat heart models reveal?

Protein levels of PP2A-C and PP2A-A

These two models showed different responses regarding protein levels during I/R. The H9c2 cells revealed an unstable PP2A-A subunit which tended to be present at reduced levels, while the isolated heart exhibited an increase in the PP2A-C subunit. Pooling of the perfusion data also showed a reduction in PP2A-A levels at 5 minutes reperfusion, similar to the reduction seen in the H9c2 cells at 15 minutes reperfusion. In both models PP2A-A returned to normal levels with prolonged reperfusion. Not much is known concerning the dynamics of the PP2A-A subunit in the heart. Penna *et al.* (2011) reported that PostC reduced PP2A-A levels as measured at 60 minutes reperfusion. Unfortunately they did not include a non-ischaemic group for comparison. Although their data shows a reduction at a different timepoint than ours, it does illustrate that the levels of the A subunit are dynamic in the heart and that its reduction during reperfusion could be beneficial. Possibly the reduction observed could be part of the pro-survival cascades activated in the heart cells in response to I/R.

These observed differences between the H9c2 cells and the isolated rat heart can obviously be ascribed to differences between these two models – and there are outspoken differences. H9c2 cells are very different from the beating heart in terms of susceptibility to ischaemic damage, metabolic requirements, exposure to inflammatory mediators, contractile characteristics and

proliferation. It would therefore be interesting, and possibly more informative, to also characterize PP2A in freshly isolated cardiomyocytes exposed to I/R.

It is however fascinating that despite these differences in total protein levels a similar trend can be seen in both models: ischaemia is associated with an increase in the ratio of PP2A-C to PP2A-A (Figures 3.6, 3.10, 3.16 and 3.18), which is abolished during reperfusion in the H9c2 cells, and from 10 minutes reperfusion onwards in the heart tissue. In fact there is a reduction in the ratio at 30 minutes reperfusion in the isolated organ model. This would indicate an increase in PP2A-A relative to PP2A-C, and at 30 minutes reperfusion might be due to a residual over-correction of PP2A-C levels. Since PP2A-A is unstable if not incorporated into the PP2A holoenzyme (Silverstein *et al.*, 2002), it would be interesting to see if this reduction in ratio persists following a longer period of reperfusion.

PP2A-C methylation

Not only were there similarities regarding the ratio of PP2A-C to PP2A-A, but the associated methylation pattern was also comparable. Whenever we see an increase in the ratio of PP2A-C/A in both models, there is also an accompanying increase in at least absolute measured nonmethylation (Figures 3.11 and 3.17), if not the ratio of nonmethylated to total PP2A-C as well (Figures 3.7, 3.18 and 3.21). Although there are instances when the nonmethylated signal increased in the absence of changes in PP2A-C/A, it is very tempting to speculate that these two observations are linked, especially since methylation influences enzyme assembly and a shift in PP2A-C/A could be an indication of trimer disassembly. Disregarding the possible link between PP2A-C/A and methylation, our data shows that ischaemia is associated with a reduction in methylation (increase in nonmethylation) in both the H9c2 cells, as well as isolated heart tissue. In our *ex vivo* rat heart preparation we also found that this reduction in methylation persisted for at least the first 10 minutes of reperfusion.

To my knowledge nothing is known regarding methylation in the heart exposed to I/R. Consistent with ischaemia inducing PP2A demethylation, Lee and co-workers (2013) found that an 18 hour exposure of a neuroblastoma cell line to a mitochondrial complex I inhibitor, MPP^+ (1-methyl-4-phenylpyridinium), led to the demethylation of PP2A. In agreement with our tissue reperfusion data, De la Vega and colleagues (2002) also reported reperfusion-associated demethylation of PP2A in an *in vivo* rat model of induced forebrain ischaemia and reperfusion. They also reported that methylation of PP2A-C was associated with the formation of the trimeric holoenzyme. As discussed previously, this is dependent on the type of B-subunit present. Interestingly, and contradictory to our data, Benziane *et al*. (2012) found that the activation of AMP-activated protein kinase (AMPK) induced the methylation of PP2A in L6 myotubes. Since AMPK activation is also a feature of ischaemia (Hopkins *et al.*, 2002; Hardie, 2004), this implies that PP2A might be

methylated due to activated AMPK during ischaemia. However, as already discussed in Chapter 1, ischaemia is much more complex than just one kinase.

PP2A-C phosphorylation

We hypothesised that the activity of PP2A will either remain the same, or increase, as ischaemia progresses, followed by a reduction in activity at the onset of reperfusion. We utilized tyr307 phosphorylation of PP2A as a readout of its activity, since it has been reported that the activity of PP2A is reduced in conjunction with phosphorylation of this residue (Chen *et al.*, 1992).

This expectation was met in the H9c2 cell model, where SI failed to elicit any response with regards to the phosphorylation of PP2A-C. Only at 15 minutes reperfusion a reduction in the absolute levels of phosphorylated PP2A-C was seen (Figure 3.13) which might imply an unexpected increase in activity during reperfusion. The physiological impact of this increase is uncertain, since it was not robust enough to change the level of phosphorylation relative to total PP2A-C. The heart tissue, on the other hand, revealed an increase in absolute phosphorylation at 20 minutes GI (Figure 3.18), which persisted and increased in strength into early reperfusion so that the ratio of phosphorylated to total PP2A-C also increased (Figures 3.21 and 3.22). This indicates a robust reduction in PP2A activity, which is unexpected at the end of ischaemia, but supports the suggestion that adaptive endogenous pro-survival kinase-mediated pathways dominate during early reperfusion (provided ischaemia was not too lethal).

A literature search on the response of PP2A to I/R, yielded controversial results. On the one hand it has been found that the activity of PP2A increases during ischaemia (Kunjan *et al.*, 2013) and also during reperfusion (Zhao *et al.*, 2008), while others have found that these events inhibit PP2A (Ho *et al.*, 2003; Zhu *et al.*, 2012). All these studies however focussed on I/R of the brain where a wide variety of protocols were followed. Kunjan and colleagues (2013) found that PP2A was activated in isolated rat hippocampal synaptosomes exposed to 60 minutes of oxygen and glucose deprivation; similarly Zhao *et al.* (2008) reported peak activation of PP2A at 1 hour reperfusion following 10 minutes of forebrain ischaemia in a rat model. On the other hand Ho and colleagues (2003) reported a reduction in PP2A activity in rat brains at 30 minutes reperfusion following 8 minutes of asphyxia; while Zhu *et al.* (2012) noted a reduction in PP2A levels and activity 24 hours after a 1 hour occlusion of the middle cerebral artery in a rat model. There is however nothing known in the context of the heart. Studies done in the setting of oxidative stress have also reported that PP2A is either activated (Pham *et al.*, 2000; Yasuoko *et al.*, 2004; Liu & Hofmann, 2004) or inhibited (Zheng *et al*., 2003; Jung *et al.*, 2013).

It is very interesting to note that most of the changes observed at the end of ischaemia (increased nonmethylation, increased PP2A-C/A ratio and increased phosphorylation) persisted into

reperfusion and were not reversed by alleviation of ischaemia. It is possible that these changes are part of a general response to cell stressors, or it could be that the temporal dynamics of reperfusion extend past the timepoints which we investigated.

Connecting the dots

Although some of the data generated in the two experimental models differed from each other, the following model seems to emerge from the combination of these results (Figure 3.56): Especially during ischaemia the ratio of PP2A-C to PP2A-A increases, indicating that in comparison to control conditions there is an increase in PP2A-C relative to PP2A-A. Since they associate in a 1:1 ratio in both the core- and holoenzyme complexes, this indicates that there must be additional PP2A-C present in the cells. Although it seems that PP2A-A becomes unstable, especially during the onset of reperfusion – indicating the possible disassembly of the enzyme complex – there are no indications of a reduction in PP2A-C levels. This implies that PP2A-C must be binding to a stabilising factor. The reduction in methylation hints at the factors which might be involved. Demethylation itself will require association with PME-1. PME-1 has been shown to stabilise an inactive form of the enzyme, however it does not compete with PP2A-A for binding to PP2A-C. It therefore seems more likely that PP2A-C binds to α4. As already mentioned elsewhere, α4 preferentially binds nonmethylated and dephosphorylated PP2A-C (Chung *et al.*, 1999; Kong *et al.,* 2009; McConnel *et al.*, 2010). This implies that the phosphorylated PP2A-C which we measured might be an inactive form of the core- or holo-enzyme, presenting yet another potential store of PP2A inside the cells.

But why the association with α4? It could be part of a mechanism to transiently deactivate PP2A-C during ischaemia and especially reperfusion (linking with our phosphorylation data) without reducing its actual levels in the cell. Binding to α4 is in this sense ideal, since it could theoretically regulate the rate of degradation of PP2A-C (linking to the increase in PP2A-C which we observed in the heart tissue). It could also be that α4 targets PP2A-C to specific substrates and areas within the cell, without necessarily inactivating the enzyme.

In view of the above, we speculate that cardiomyocytes keep a potentially inactive store of PP2A during I/R and that the degree and duration of ischaemia will influence the utilization of this store – possibly as ischaemia progresses and cells become committed to apoptosis these stores of PP2A-C can be utilized to mediate the process.

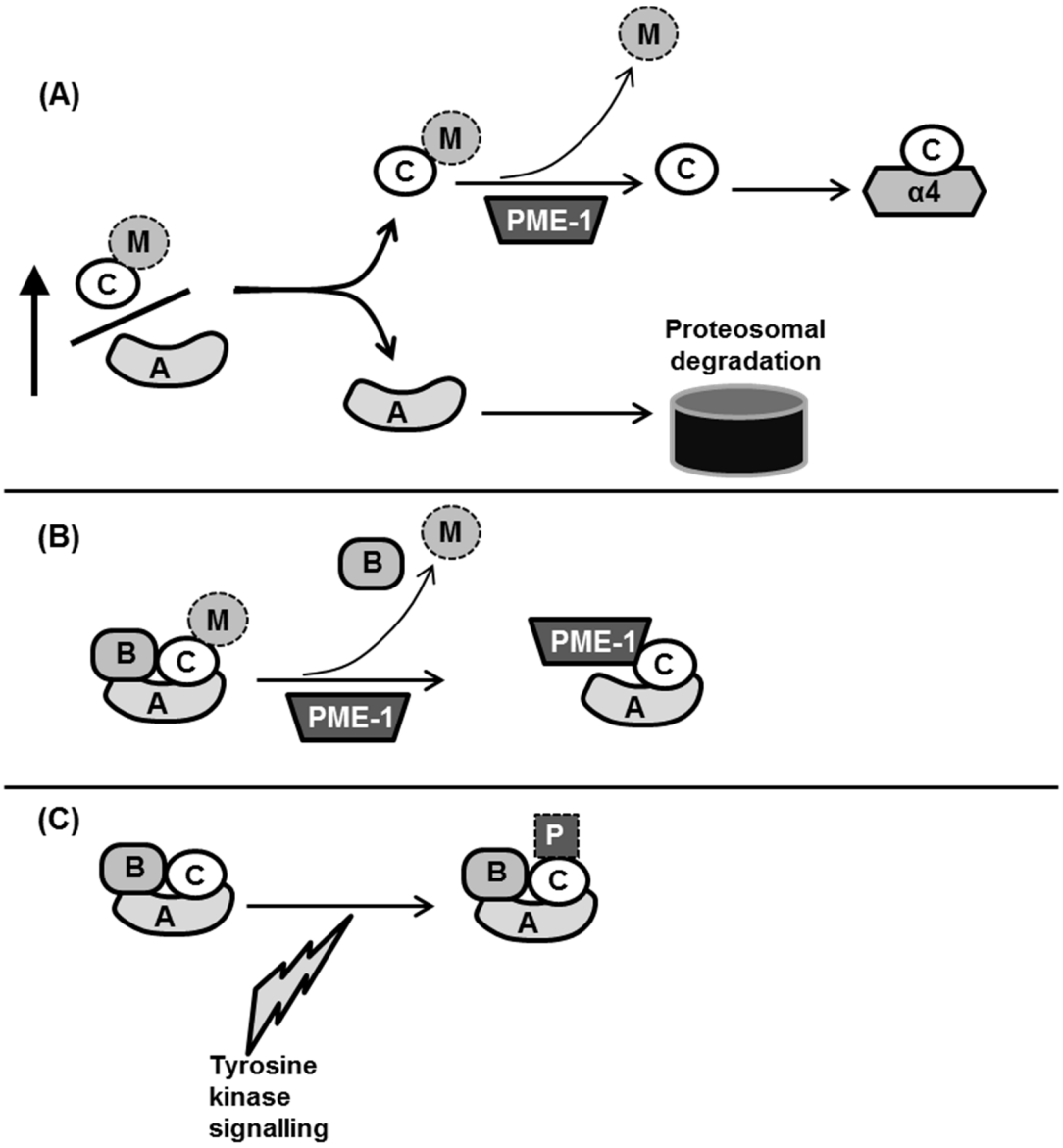


Figure 3.56. The possible ways in which cardiomyocytes store inactive PP2A during ischaemia and early reperfusion.
Our data suggest three possible ways in which PP2A is stored inside the cardiomyocyte: (A) PP2A-C dissociates from PP2A-A and is demethylated by PME-1 whereafter it binds to α4; (B) PP2A is demethylated by PME-1 whereafter it remains bound in an inactive complex with PME-1; (C) the holoenzyme is simply phosphorylated, thereby transiently inactivating PP2A.

In our experimental model, this inhibition of PP2A-C at the end of ischaemia and the onset of reperfusion could be beneficial, since it will allow the activation of cellular processes which are usually inactivated by PP2A-C, contributing to survival.

In view of the reduction in methylation of PP2A-C during I/R, it is very possible that PP2A-C translocates inside the cells as ischaemia and reperfusion progress. To investigate the intracellular dynamics of PP2A-C, we also investigated its levels and posttranslational modifications in crude nuclear, cytosolic and membrane fractions.

The cellular distribution and posttranslational modification of PP2A during ischaemia and reperfusion.

For these experiments we only used tissue collected from the isolated rat heart experiments to prepare nuclear, cytosolic and membrane fractions, in which we measured total PP2A-C, PP2A-A, nonmethylated PP2A-C and phosphorylated PP2A-C. We can therefore only speculate on the actual holoenzyme composition and substrate targeting in these different fractions. For a helpful review concerning the distribution of regulatory B subunits, as well as the factors involved in enzyme assembly, see Janssens *et al.* (2008). Each fraction will be discussed with regards to PP2A changes throughout the latter part of ischaemia, as well as early reperfusion.

The nuclear fraction

This fraction proved to be the most interesting fraction (at least at 10 minutes of GI). At this relatively early stage of ischaemia there was an accumulation of PP2A-C in the nucleus, associated with an increase in PP2A-C/A (Figure 3.25), absolute phosphorylation and nonmethylation. However the increase in total PP2A-C was of such a magnitude that most of the PP2A-C present was unphosphorylated and methylated (Figure 3.26).

The increase in absolute phosphorylation and nonmethylation implies that a portion of the PP2A-C might be associated with either α4, or PME-1. This is an attractive idea, since it explains why we did not measure a similar increase in PP2A-A. PME-1 has been shown to be abundant in the nucleus (Longin *et al.*, 2008), while α4 has been implicated in targeting PP2A to the nucleus (Fielhaber *et al.*, 2009). However, the majority of the accumulated PP2A-C seems to be methylated (Figure 3.26) – thereby excluding PME-1 and α4 as possible binding-partners. There are also no indications of an accumulation of a matching amount of PP2A-A for the formation of a canonical holoenzyme. This implies an association with an unknown binding-partner besides PP2A-A, α4 or PME-1. The endogenous inhibitors of PP2A, I_1^{PP2A} and I_2^{PP2A} can both be localised to the nucleus (Santa-Coloma, 2003). This means that even some of the unphosphorylated PP2A-C might be inactive. It has been shown that I_2^{PP2A} can also bind PP2A-C alone (Li *et al.*, 1996). Our data therefore indicates an accumulation of PP2A-C in the nucleus, probably bound to several scaffolds (methylated to PP2A-A, nonmethylated to α4) and inhibitors (α4, PME-1, I_1^{PP2A} or I_2^{PP2A}).

Concerning the portion of PP2A-C assembled with PP2A-A into a holoenzyme, the presence of such a large proportion of methylated PP2A-C suggests that the holoenzyme will probably also contain B/PR55 regulatory subunits, which can target PP2A to the nucleus. Other regulatory subunits which have been found in the nucleus (B''/PR72 and B' / B56γ and B56δ) are not as methyl-dependent as B/PR55. B/PR55 also preferentially binds unphosphorylated PP2A-C, therefore implicating it even further.

Such a transient accumulation must have some functional implications inside the cell. Several possible targets for PP2A inside the nucleus have been identified. Zhou and colleagues (2007), following a proteomic approach to identify some of the binding-partners of B56γ1, identified approximately 16 binding-partners located in the nucleus. Firulli *et al.* (2003) reported that PP2A was involved in the dephosphorylation of the transcription factor HAND1, while Ling *et al.* (2012) recently showed that PP2A can interact with and dephosphorylate histone deacetylase 4. It is therefore very conceivable that PP2A accumulation in the nucleus will contribute to the regulation of nuclear function and transcription, possibly as part of a relatively early adaptive response to ischaemia.

At 15 minutes GI the accumulation of PP2A-C was abolished and no changes relative to control were measured (Figures 3.31 and 3.32). Late in ischaemia the absolute phosphorylation of PP2A-C increased (Figure 3.39) indicating a reduction in activity which becomes more robust at 5 minutes reperfusion (Figure 3.45). By 10 minutes reperfusion all parameters measured in the nucleus are comparable to control levels.

In summary: In the first half of ischaemia (after 10 minutes GI) PP2A is part of a nucleus-based, probably transcription-related, response to ischaemia. This raises the question whether PP2A is in some or other way also involved in late preconditioning (SWOP) which is dependent on protein expression to mediate its protective effects. This initial response is however soon replaced by the inhibition of PP2A-C later in ischaemia and at the very early stages of reperfusion (5 minutes) – similar to the trend observed in the “complete cell” unfractionated preparations.

The cytosolic fraction

It is only at 15 minutes GI that a response to ischaemia was seen, which was in the form of an increase in total PP2A-C (Figure 3.33) with an associated reduction in phosphorylation (Figure 3.34). There was no increase in the ratio of PP2A-C to PP2A-A. Taken together, this implies an increase in active PP2A-C in the cytosol. Almost all of the regulatory subunits have been found to be present in the cytosolic fraction, including B/PR55, B’’/PR72 and B’/PR61. Since we did not see any changes in methylation, it does not give us information regarding the identity of the specific regulatory subunit(s) involved.

For all the other time periods investigated, no evidence for any form of posttranslational modification was obtained. Interestingly, despite the absence of changes in methylation, a consistent increase in the PP2A-C/A ratio during both ischaemia and reperfusion was observed.

In summary, it seems as if the nuclear accumulation of PP2A-C at 10 minutes GI is followed by an increase of PP2A-C in the cytosolic fraction at 15 minutes GI. For the rest of the experimental

duration there is, possibly, a residual increase in the amount of PP2A-C relative to PP2A-A. This small portion of excess PP2A-C will necessarily have to be bound to α4. In the absence of changes in posttranslational modification we cannot speculate on which binding partners are involved. Initially most of the PP2A-C present is in an active form, although as ischaemia progresses and during early reperfusion the levels of phosphorylation do not differ from the control levels.

The membrane fraction

At 10 minutes GI there is a borderline reduction in PP2A-C levels (Figure 3.29), suggesting that some of the PP2A-C accumulated in the nucleus at this time might have originated from the membrane fraction. As explained in the results section, the cytosolic and membrane fractions contain much larger amounts of PP2A-C than the nucleus, so that small changes in these fractions could be associated with a major change in PP2A levels in the nucleus.

The level of methylation fluctuates substantially during I/R; at 10 minutes GI there is a reduction in methylation, at 20 minutes GI an increase in methylation and then again a reduction at 10 minutes reperfusion. This suggests possible movements of PP2A-C between different regulatory subunits. B/PR55 is methyl-dependent and has been described in association with a membrane fraction, while the other membrane associated subunit, B'/PR61, has been found associated with both methylated and nonmethylated PP2A-C. If PP2A-C is indeed being shuttled between regulatory subunits, a very dynamic regulatory system must be at work here.

Following the same trend as the unfractionated samples and the nuclear fraction, the phosphorylation of PP2A increases at 5 minutes reperfusion; suggesting the inhibition of PP2A during early reperfusion.

Connecting the dots

Our data indicate that PP2A participates in a relatively early nuclear response to sustained ischaemia, as evident by an accumulation of especially dephosphorylated, methylated PP2A-C in the nucleus. Following this accumulation, PP2A-C translocates away from the nucleus towards the cytosol. The cytosol is the compartment which contains the most PP2A, and it is in this compartment where we see an increased ratio of PP2A-C to –A from 20 minutes GI, continuing through 5 and 10 minutes reperfusion. This links with the increased ratio observed in the unfractionated preparations. Contrary to expectations, we did not observe the same associated changes in methylation, as was the case for the unfractionated samples. In fact, we failed to pin down the position of the nonmethylated PP2A-C which we found to be abundant in the "whole cell" preparations. At 20 minutes GI and especially 5 minutes reperfusion it seems that most of the phosphorylated PP2A-C is localized in the nuclear, as well as the membrane fractions.

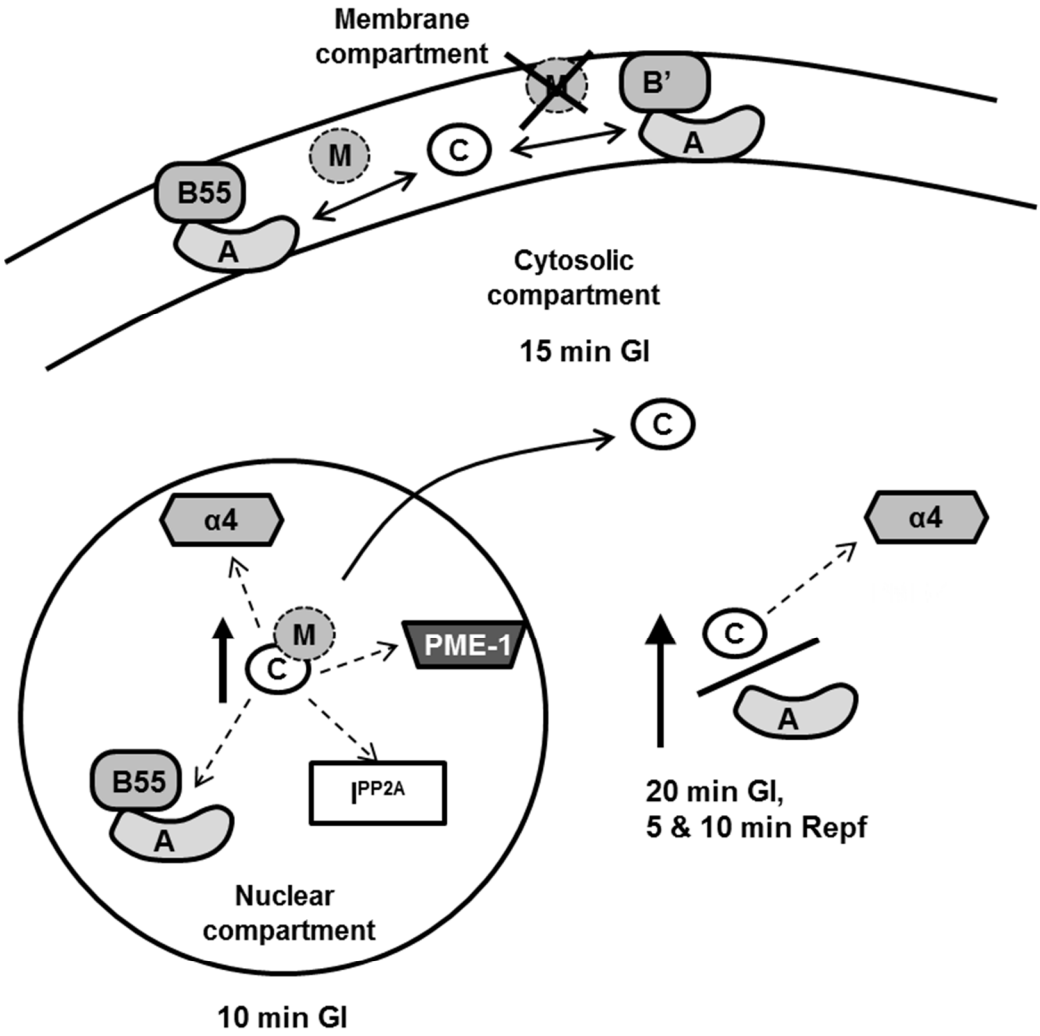


Figure 3.57. Distribution of PP2A during ischaemia and early reperfusion. Our data indicates an accumulation of PP2A-C in the nuclear fraction at 10 minutes GI, whereafter PP2A-C translocates to the cytosolic fraction. The cytosolic fraction is also characterized by a persistant increase in PP2A-C/A during late ischaemia and early reperfusion. In the membrane fraction methylation is very dynamic, suggesting dynamic holoenzyme assembly.

Given the obvious differences in the preparation of the fractionated versus the unfractionated samples, the fact that each fraction contributes differently to the total pool of PP2A and the additional complexity due to the increase in PP2A-C levels and reduction in PP2A-A levels at 5 minutes reperfusion in the unfractionated preparations, it is not surprising that there are differences between these data sets which we cannot explain.

Although the membrane fraction did not participate in the translocation of PP2A-C, there seems to be a dynamic methylation-dependent mechanism at work, potentially targeting PP2A to different substrates within this fraction as ischaemia and reperfusion progresses.

A summary of the model which our data suggests is given in Figure 3.57.

Limitations and future directions

In my view, the major outcomes of this study are: (1.) It provides definite proof that PP2A is recruited into the cellular response to I/R, thereby identifying PP2A as a role-player in this setting which needs to be further investigated; and (2.) It forms a basic lattice-work for future studies. We only described the basics concerning PP2A, without investigating any of the role-players involved in its regulation, for example PME-1 and α4. These are all aspects which still require further investigation.

Future directions include further experimentation to confirm our results, as well as to expand on what has already been done. With regards to confirmation the following can still be done:

1.) As mentioned earlier in this text, the ongoing proliferative capacity of the H9c2 cells is a potential confounding factor, since PP2A has been implicated in the control of the cell cycle. One possible way in which to minimize this effect would be to chemically differentiate the cells prior to experimentation. This entails incubating the cells for a period of 4-6 days in medium containing low levels of serum (1% vs the normal 10% used here), combined with a relatively low concentration of retinoic acid (10 nM). Under these conditions H9c2 cells have been shown to differentiate into cells with an increased expression of cardiac specific proteins and a markedly reduced proliferative capacity (Ménard *et al.*, 1999; Pagano *et al.*, 2004; Pereira *et al.*, 2011; Branco *et al*., 2012). It would therefore be advantageous to repeat our SI/R experiments in such a cardiac-specific differentiated H9c2 cell model.
2.) Investigation of the increase in total PP2A-C in unfractionated lysates at 20 minutes GI. This can be done by measuring both the mRNA levels of PP2A-C, as well as determining the level of PP2A-C ubiquitination as ischaemia progresses. Investigating the effects of proteosomal inhibition during ischaemia and reperfusion will also be very insightful.
3.) The fractionation data revealed some interesting and sometimes surprising results, especially the nuclear accumulation of PP2A-C at 10 minutes GI. The fractionation procedure should be improved to yield pure sarcolemmal, mitochondrial, sarcoplasmic reticulum and myofibrillar fractions. It would also add value if the intracellular distribution of PP2A-C is investigated in the H9c2 cell model using immunofluorescence microscopy and live cell imaging.

In addition to the above, the following aspects should be investigated:

1.) Identification of the substrates targeted by PP2A-C in the nucleus at 10 minutes GI. This could be addressed by pharmacological modulation of PP2A-C combined with phospho-proteomic analysis of the nuclear fraction at this time point.
2.) We only focussed on methylation and phosphorylation of PP2A-C as indications of its regulation. There are however several important proteins in the regulation of PP2A-C which

should receive attention in the context of myocardial I/R. These include PME-1, LCMT-1, α4, TIPRL and PTPA.

3.) In our quest of a basic description of PP2A, we only focussed on the components of the core enzyme: PP2A-A and PP2A-C. The regulatory B subunits are however the determinants of cellular location and substrate specificity. It will be very interesting to determine which regulatory subunits are involved where and when in this setting.

4.) The characterization of PP2A could also be extended beyond 10 minutes of reperfusion, and even beyond 30 minutes reperfusion. As mentioned before, DeGrande *et al.* (2013) and others have found that myocardial hypertrophy and failure are associated with elevated PP2A levels. It could be interesting to see whether PP2A is a molecular link, or simply a component, of the continuum between infarction and the development of post-MI heart failure.

Conclusion

Ischaemia elicits a mismatch between PP2A-C and PP2A-A, in such way that there is an increase in the levels of PP2A-C relative to PP2A-A. This surplus PP2A-C is especially noticeable in the cytosolic fraction and is probably stabilised in the cell, possibly through binding to a scaffold or inhibitor, such as α4. Late ischaemia and early reperfusion is associated with an increase in the phosphorylation of PP2A-C, which suggests its inhibition – thereby favouring the activation of adaptive pro-survival cellular events. This inhibition of PP2A was evident in both the nuclear and membrane fractions.

The nucleus hosted an accumulation of dephosphorylated, methylated PP2A-C at 10 minutes ischaemia. The reason for this is unknown, although PP2A probably participates in a nuclear response to ischaemia, including aspects such as changes in protein expression, etc.

The membrane fraction also revealed a dynamic situation in which the methylation of PP2A-C fluctuates throughout ischaemia and early reperfusion, implying dynamic changes in substrate specificity throughout this period.

After 10 minutes of reperfusion the levels and distribution of PP2A-C seemed to be almost back to normal, except for the elevation of PP2A-C/A in the cytosolic fraction. Posttranslational modifications however persisted at 10 minutes reperfusion.

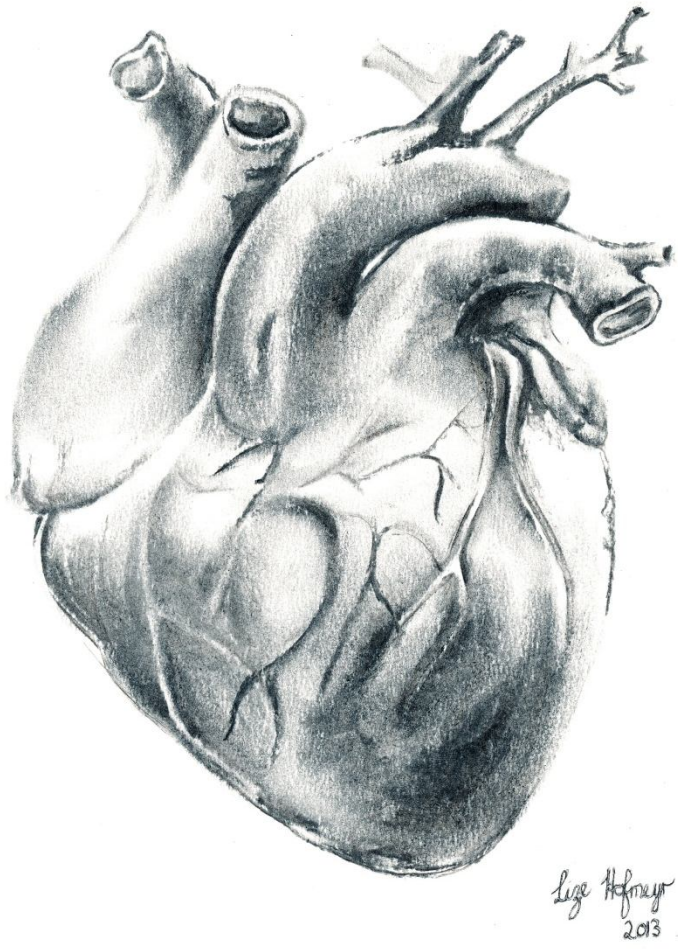
We have now shown that PP2A is involved in cellular response to myocardial I/R. The next question however is, how important is PP2A in contributing to the outcome of an I/R episode? Otherwise put: could the manipulation of PP2A significantly change the outcome of I/R injury?

CHAPTER 4

Pharmacological modulation of PP2A: Inhibition

"Greebo had spent an irritating two minutes in that box. Technically, a cat locked in a box may be alive or it may be dead. You never know until you look. In fact, the mere act of opening the box will determine the state of the cat, although in this case there were three determinate states the cat could be in: these being Alive, Dead, and Bloody Furious."

Terry Pratchett
Lords and Ladies



Chapter 4

Pharmacological modulation of PP2A: Inhibition

Introduction

As discussed in Chapter 1, possibly one of the greatest benefits of the considerable research effort which has been directed at cardioprotection, is the delineation of the signalling cascades which are involved in the heart's response to an I/R episode. We know now that cardioprotection is mediated by a vast network of interrelated signalling cascades communicating information regarding stress and adaptation from cell membrane receptors and cellular sensors to end-targets, of which the most important seems to be the mitochondria and the nucleus. For complete reviews concerning these cardioprotective signalling pathways see Yellon & Downey (2001), Hausenloy & Yellon (2007) and Yang *et al.* (2010).

Although several signalling pathways are involved in the cardiac response to I/R, cardioprotection has become almost synonymous with the RISK pathway and MAPK signalling. We were therefore interested to know what the relationship, if any, is between these signalling pathways and PP2A within the setting of I/R. This mini-review will therefore only focus on what is currently known regarding these pathways within the context of I/R, as well as the interactions which have been described between PP2A and these pathways.

Signalling in ischaemia/reperfusion and cardioprotection

The Reperfusion Injury Salvage Kinases (RISK) pathway was originally coined by Hausenloy & Yellon in 2004 as a collective concept encompassing both the PI3-kinase – PKB/Akt and ERK p42/p44 pathways. They made the observation that IPC induced the activation of both PKB/Akt and ERK p42/p44 during the trigger phase of IPC (i.e. prior to ischaemia), as well as during the onset of reperfusion (Tong *et al.*, 2000; Uchiyama *et al.*, 2004; Fryer *et al.*, 2007; Hausenloy *et al.*, 2004 & 2005). Activation of these pathways has also been implicated in the cardioprotection elicited by pharmacological PostC, as well as ischaemic PostC (Yang *et al*., 2004; Bopassa *et al.*, 2006; Bell *et al.*, 2007). The importance of these pathways is underpinned even more by the work of Sivaraman *et al.* (2007) who found that PostC protection in human atrial tissue was dependent on the activation of the RISK pathway.

It must however be noted, that the traditional components of the RISK pathway (PKB/Akt and ERK p42/p44 mediated signalling) are not the only signalling pathways implicated in survival and cardioprotection. In 2005 Lecour and colleagues identified the reperfusion associated activation of STAT3 as a mediator of cardioprotection independent of PKB/Akt and ERK p42/p44. STAT3

therefore presents an alternative cardioprotection pathway which they coined the SAFE (Survivor Activating Factor Enhancement) pathway (Lecour, 2009).

For simplicity and specificity sake, we will however limit our focus on components of the RISK and MAPK pathways.

The PI3-kinase – PKB/Akt pathway

The phosphorylation and activation of PI3-kinase and its downstream target PKB/Akt is associated with I/R (Armstrong, 2004). Cardioprotective interventions, such as IPC, PostC and their pharmacological mimetics stimulate an even further increase in the phosphorylation of PKB/Akt. Most studies have found that the protection induced by these interventions is dependent on this increased phosphorylation and activity of PKB/Akt, so that inhibition of the PI3-kinase – PKB/Akt pathway abolishes protection (Yang *et al.*, 2004; Tillack *et al.*, 2006; Fujita *et al.*, 2007). Several downstream effectors of PKB/Akt which are involved in cardioprotection have been identified and include signalling proteins such as endothelial nitric oxide synthase (eNOS) (Tsang *et al.*, 2004; Zhu *et al.*, 2006), mTOR (Hernández *et al.*, 2011) and GSK-3β (Zhu *et al.*, 2006; Tillack *et al.*, 2006). This PI3-kinase – PKB/Akt mediated phosphorylation of GSK-3β is especially noteworthy, since the phosphorylation and associated inhibition of GSK-3β is thought to mediate the stabilisation of the mPTP in a closed conformation (Juhaszova *et al.*, 2004; Bopassa *et al.*, 2006). As discussed in Chapter 1, the mPTP is regarded as one of the more, if not the most, important end-effector which mediates the balance between survival and injury following I/R.

Despite all the data which has implicated PI3-kinase – PKB/Akt as a key mediator of cardioprotection, it must also be noted that not all studies have shown a causal link between this pathway and protection (Darling *et al.*, 2005; Schwartz & Lagranha, 2006). In their isolated rabbit heart model, Darling and colleagues (2005) found that PostC protection was neither associated with an increase in PKB/Akt phosphorylation, nor alleviated by the inhibition of PI3-kinase. This study however confirmed the cardioprotective role of the other component of the RISK pathway: ERK p42/p44.

ERK p42/p44 signalling

The other “leg” of the RISK pathway is the MEK1/2 (MAPK/ERK kinase 1/2) – ERK p42/p44 pathway which, as the name denotes, is a member of the larger MAPK family which also includes c-Jun NHP_2-terminal kinase (JNK) and p38 MAPK. In the absence of a cardioprotective intervention, early reperfusion following ischaemia leads to an increased phosphorylation and activation of ERK p42/p44 (Armstrong, 2004) in a pattern that is very similar to PKB/Akt. Cardioprotective interventions enhance this phosphorylation and inhibitor studies targeting the

upstream MEK1/2 have also confirmed that ERK p42/p44 acts as a mediator of cardioprotection (Yang *et al.*, 2004; Darling *et al.*, 2005; Fujita *et al.*, 2007; Hausenloy & Yellon, 2007).

It has also been suggested that ERK p42/p44 phosphorylates GSK-3β, thereby promoting maintenance of the mPTP in a closed conformation (Juhaszova *et al.*, 2004; Hausenloy & Yellon, 2007).

As is the case with PKB/Akt, there is no consensus in the literature regarding the importance of ERK p42/p44 in mediating protection. In this regard, Feng and co-workers (2006) reported that isoflurane PostC, though dependent on PI3-kinase – PKB/Akt signalling, is not associated with ERK p42/p44 phosphorylation. In an intriguing study by Schwartz & Lagranha (2006) a PostC protocol induced the phosphorylation of both PKB/Akt and ERK p42/p44, but failed to elicit an infarct sparing effect.

A possible explanation for these disparate results is hinted at by a study done by Hausenloy *et al.* (2004) which indicated the existence of cross-talk between the PI3-kinase – PKB/Akt and MEK1/2 – ERK p42/p44 pathways in such a way that inhibition of the one pathway was associated with an increase in the phosphorylation of the other pathway. Application of the principle indicated by this study to the myocardial context, where multiple pathways mediating protection are activated by an ischaemic conditioning intervention, suggests a situation where eventual protection is mediated by more than one pathway. The implication of this is that the inhibition of a single pathway may not abrogate protection, while activation of only one or two of these pathways is not necessarily enough to cross the threshold of protection.

Despite these conflicting results, the current paradigm is that the activation of PKB/Akt and ERK p42/p44 is involved in mediating cardioprotection. It is also noteworthy that both these kinases signal on to GSK-3β, placing it as a central regulator in cardioprotection.

Another member of the MAPK family which has received a large amount of attention, but with much less unequivocal results is p38 MAPK.

p38 MAPK in cardioprotection

As a member of the MAPK family it seems obvious that p38 MAPK would be a mediator of cardioprotection. However, its varied and intricate roles in survival and apoptosis excludes such a straightforward answer.

In contrast to ERK p42/p44 and PKB/Akt, p38 MAPK phosphorylation already increases during sustained ischaemia, peaking during early reperfusion (Armstrong *et al.*, 1998; Ma *et al.*, 1999;

Armstrong, 2004). In 1997 Weinbrenner and colleagues reported that an IPC protocol applied to the isolated rat heart induced an increase in the ischaemic phosphorylation of p38 MAPK. Combined with the observations that this activation was linked to protection and the inhibition of p38 MAPK abolished cardioprotection, the authors concluded that p38 MAPK activation is an important component of IPC protection. Similarly Armstrong and co-workers (1999) reported that IPC increased the phosphorylation of p38 MAPK during ischaemia in conjunction with protection. The importance of p38 MAPK was also shown to extend beyond ischaemia, with Da Silva *et al.* (2004) reporting that IPC elicited an increase in p38 MAPK phosphorylation after 40 minutes of global ischaemia and 30 minutes reperfusion in an isolated rat heart model. Others have also implicated p38 MAPK phosphorylation during I/R as an important component of IPC (Nagy *et al.*, 2007; Hernández *et al.* 2011), as well as other cardioprotective interventions such as dietary supplementation with red palm oil (Engelbrecht *et al.*, 2006) and insulin administration during reperfusion (Tiron *et al.*, 2006). There are however other studies which have shown the opposite, namely that p38 MAPK activation and phosphorylation during sustained ischaemia and reperfusion are detrimental to the heart (Ma *et al.* 1999; Mackay & Mochly-Rosen *et al.*, 2000; Kim *et al.*, 2012; Thomas *et* al., 2011; Surinkaew *et al.*, 2013). For a review about the inconsistencies concerning the research which has been done regarding the role of p38 MAPK in cardioprotection see Steenbergen (2002).

So, ultimately the question remains: Is p38 MAPK activation protective or detrimental? In his review on the topic Steenbergen (2002) lists some of the confounding factors which could explain the controversy, including animal species differences, issues with the pharmacological inhibitors used in these studies and the question regarding the specific p38 MAPK isoforms involved. Early studies did not take this latter variable into account, but it is a relevant issue since the two prevalent isoforms in the heart (α and β) exert different effects, as exemplified in a study by Wang *et al.* (1998) which showed that p38α MAPK seems to induce apoptosis, while p38β MAPK favours hypertrophic remodelling. In this regard, Saurin *et al.* (2000) found that IPC protection is dependent on the inhibition of specifically p38α during sustained ischaemia. For a review concerning the importance of the p38 MAPK isoforms in myocardial I/R see Bassi and colleagues (2008). Another aspect which must be kept in mind concerns the temporal characteristics of p38 MAPK activation in and around cardioprotection.

Research done in our laboratory placed the pro-survival aspect of p38 MAPK activation within the triggering phase of IPC, i.e. during the ischaemic conditioning stimulus prior to sustained ischaemia (Marais *et al.*, 2001 and 2005). In this model IPC elicits an increase in the activity of p38 MAPK during the triggering stimulus, but reduces it during subsequent sustained ischaemia and reperfusion. The cardioprotective importance of p38 MAPK activation during the IPC protocol itself has also been shown by Da Silva *et al.* (2004).

The precise role and contribution of p38 MAPK to I/R injury and cardioprotection is therefore still an open book, but that it is somehow involved in these processes is however unquestionable.

The role of PP2A in intracellular signalling

Having briefly described the current state of knowledge regarding the participation of PKB/Akt, ERK p42/p44, GSK-3β and p38 MAPK in cardioprotection, the focus will now shift to the interaction between PP2A and these signalling molecules. Due to the limited information available concerning the involvement of PP2A in cellular signalling in the heart, data generated in other tissues and models will also be included in this mini-review.

p38 MAPK: activator or effector of PP2A?

In 1995 it was already reported that p38 MAPK can be dephosphorylated, and in the process inactivated, by PP2A in rat PC12 cells (Doza *et al.*, 1995). This early study also found that activation of this kinase requires simultaneous phosphorylation of both a tyrosine and a threonine residue (at positions 182 and 180 respectively). PP2A was implicated in the dephosphorylation of the threonine residue, while protein tyrosine phosphatase-1B (PTP-1B) dephosphorylated the tyrosine residue. The phosphorylation of two residues are therefore of importance here, while the dephosphorylation of these two residues can be catalyzed by any of the three families of protein phosphatases.

Mackay & Mochly-Rosen (2000) targeted the inhibition of the tyrosine phosphatases in a model of isolated rat ventricular cardiomyocytes exposed to hypoxic conditions. They found that incubation of the cells with the PTP inhibitor, vanadate, during hypoxia increased the phosphorylation of p38 MAPK compared to control and in the process also augmented the release of LDH from the cells, implying that p38 MAPK activation is detrimental. Focussing on the PSPs, Armstrong and colleagues (1998 and 1999) found that the incubation of isolated rabbit cardiomyocytes with the PP2A and PP1 inhibitor, Calyculin A, from 10 minutes prior to ischaemia through 30 minutes ischaemia (induced by pelleting the cells and sealing the pellets from atmospheric oxgen with a layer of mineral oil) was also associated with an increase in the phosphorylation of p38 MAPK. It is interesting to note that the increase in p38 MAPK phosphorylation during ischaemia in this particular model was associated with a protective effect. Juxtaposing these studies leads to the speculation that the phosphorylation of tyr182 is detrimental, while thr180 is associated with resistance against stress. Data reported in a recent study by Omar and colleagues (2012) however disprove this speculation. They found that adenosine-triggered cardioprotection is mediated by a reduction in glycolysis and proton generation during reperfusion following 17 minutes GI in an isolated working rat heart model. These metabolic effects were dependent on the dephosphorylation of both AMPK and p38 MAPK and could be abrogated by the administration of

cantharidin or okadaic acid – both inhibitors of both PP1 and PP2A. This work has three intriguing implications: (1.) p38 MAPK activity during reperfusion is detrimental; (2.) PP1 and / or PP2A can mediate protection and; (3.) PP1 and / or PP2A dephosphorylate p38 MAPK in the heart. Utilization of small T expression (a protein of viral origin which can inhibit PP2A, see the introduction to Chapter 3) to modulate and inhibit the activity of PP2A also revealed an increased phosphorylation of p38 MAPK in a mouse fibroblast cell system (Andrabi *et al.,* 2007). In T leukemia cells Boudreau and colleagues (2007) found that PP2A was involved in maintaining p38 MAPK in a hypophosphorylated state, potentially by directly interacting with the kinase. Intriguingly in their study the activity of both PP2A and p38 MAPK favoured cell survival, so that the combined inhibition of both these enzymes led to apoptosis.

These studies therefore firmly establish PP2A as an upstream regulator of p38 MAPK. There is however also a large body of work which places PP2A as a downstream effector of p38 MAPK signalling.

In one of the first demonstrations of a link between p38 MAPK and ERK p42/p44, Westermarck and colleagues (2001) reported that, in a fibroblast cell model, MAPK kinase 3 (MKK3) activation of p38α MAPK induced a reduction in the activity of the MEK1/2 – ERKp42/p44 pathway by somehow activating PP1 and PP2A, thereby mediating the dephosphorylation and inactivation of MEK1/2. Li *et al.* (2003) confirmed these results, with the only additions that p38β MAPK also induces MEK1/2 inactivation and the proposal that the relevant phosphatase is probably PP2A. Following these studies in fibroblast and cancerous cell models, the existence of this pathway was also confirmed in endothelial cells (Grethe & Pörn-Ares, 2006) and isolated adult rat ventricular myocytes (Liu & Hofmann, 2004). In both these studies the p38 MAPK-PP2A mediated dephosphorylation of the MEK1/2-ERK p42/p44 pathway favoured apoptosis following exposure to either TNF-α or oxidative stress. These studies place PP2A as the “connector” in the cross-talk between p38 MAPK and ERK p42/p44, and simultaneously also at the signalling tipping point between cell survival and apoptosis.

It is however not only ERK p42/p44 which is targeted by a p38 MAPK-PP2A mechanism. Avdi and colleagues (2002) demonstrated a similiar pathway in human neutrophils, but with the major difference that p38 MAPK activated PP2A directed against MAPK kinase 4 (MKK4), which is upstream of JNK. In this case however JNK dephosphorylation was associated with an abrogated apoptotic response to TNF-α exposure. In cardiomyocytes Zuluaga *et al*. (2007) also found evidence that p38α can induce the dephosphorylation of PKB/Akt in a PP2A dependent manner. Interestingly, the mechanism which they described implicates p38 MAPK in regulating the interaction between caveolin-1 and PP2A which is necessary for the translocation of PP2A from the nucleus to the sarcolemma where it can target PKB/Akt.

There is therefore compelling evidence identifying PP2A as both a regulator of p38 MAPK, as well as a downstream target of p38 MAPK. The only conclusion that can be made is that both these scenarios are possible, and that the type of cell as well as the stimuli affecting that cell will determine which of these pathways are recruited.

ERK p42/p44: Inhibited or activated by PP2A?

As already touched upon in the previous section, PP2A has been associated with the dephosphorylation and inactivation of ERK p42/p44. In fact, this link was identified many years ago. As decribed for p38 MAPK, full activation of ERK p42/p44 requires the phosphorylation of both a tyrosine, as well as a threonine residue. In 1990 Anderson and colleagues reported that ERK p42/p44 could be deactivated by treatment with either the tyrosine phosphatase CD45, or PP2A. Since then several studies have shown that PP2A is involved in the dephosphorylation of ERK p42/p44 (Sontag *et al.*, 1993; Alessi *et al.*, 1995; Quintaje *et al.*, 1996; Nyunoya *et al.*, 2005). In fact Alessi and colleagues (1993) suggested that PP2A might be the major factor involved in the deactivation of ERK p42/p44. It is also important to note that Quintaje *et al.* (1996) demonstrated this interaction between PP2A and ERK p42/p44 in rat neonatal cardiomyocytes, implicating PP2A as a negative regulator of ERK p42/p44 in the heart. This was also demonstrated by Taglieri *et al.* (2011) who showed that p21-activated kinase-1 (Pak-1) can inhibit ERK p42/p44 in the heart in a PP2A dependent manner.

Although PP2A has been identified as the primary direct deactivator of ERK p42/p44, it seems that the interaction of PP2A with the broader ERK p42/p44 pathway is more complex. In the extended pathway, activation of Ras leads to the plasma membrane localization and activation of the kinase Raf-1, which in turn can phosphorylate and activate MEK1/2, which then activates ERK p42/p44. The activation of Raf-1 is a complex procedure which requires multiple phosphorylations, transient interaction with the scaffold 14-3-3 and, importantly for this discussion, dephosphorylation of the inhibitory phospho-residues serine 259 and 621. Work done by several researchers have revealed that PP2A is involved in the dephosphorylation of the inhibitory serine 259 residue, implicating PP2A as an activator of Raf-1 and therefore also of the MEK1/2-ERK p42/p44 pathway (Jaumot & Hancock, 2001; Abraham *et al.*, 2001; Adams *et al.*, 2005). In addition to this, Ory and colleagues (2003) also showed that PP2A-mediated dephosphorylation of serine 392 in the scaffold protein KSR1 (kinase suppressor of Ras 1) is important for the successfull KSR1 mediated interaction of MEK1/2-ERK p42/p44 with Raf-1 at the cell membrane. PP2A is therefore involved as a positive regulator at two points in the pathway in the eventual activation of ERK p42/p44.

We are therefore left with two disparate conclusions regarding PP2A and ERK p42/42, namely that PP2A is both an activator, as well as a deactivator of ERK p42/p44. The obvious question which emerges is how are these two roles reconciled in one cell? One possible answer is alluded to by

Adams *et al.* (2005) who identified the PP2A holoenzymes which target and activate Raf-1 as containing either Bα or Bδ. In their study Sontag *et al.* (1993) found that small T expression failed to exert an effect on Raf-1, although it inhibited the ability of PP2A to dephosphorylate MEK and ERK. This lack of an effect on Raf-1 makes sense though when you realize that in their model small T exchanged with Bα, thereby reducing the abundance of this specific PP2A holoenzyme. As discussed in Chapter 3, the regulatory B subunit is important in determining both the cellular location as well as substrate specificity of PP2A. It is therefore very probable that in this situation different regulatory subunits are involved in targeting PP2A to either Raf-1, RSK1 or ERK p42/p44. The type of B subunit involved is however probably not the only factor involved, since Van Kanegan *et al.* (2005) showed that in PC3-6 cells Bα and Bδ also directly targeted PP2A to ERK p42/p44, exactly the same regulary subunits as linked to the dephosphorylation of Raf-1 (Adams *et al.*, 2005). No doubt other factors, such as cell type and stimulus involved, are also important in determining the role of PP2A in ERK p42/p44 signalling.

Another twist in the interaction between ERK p42/p44 and PP2A has been revealed by Letourneux and colleagues (2006). They found that the protein IEX-1 (or ier3) can protect ERK p42/p44 from PP2A dephosphorylation by docking ERK p42/p44 and the B56γ1 containing PP2A holoenzyme in the same ternary complex. When associated in this complex ERK p42/p44 has an increased activity towards the B56 subunit. ERK p42/p44 mediated phosphorylation of B56 however triggers the dissociation of PP2A-C from the holo-enzyme. IEX therefore serves as a scaffold allowing for the ERK p42/p44 mediated inhibition of PP2A activity directed against ERK p42/p44, as well as PKB/Akt (Rocher *et al.*, 2007). It must however be noted that this mechanism was identified in several experimental cell models and to my knowledge has not yet been identified in the heart.

PP2A negatively regulates the PKB/Akt – GSK-3β pathway

In contrast to the MAPKs, research has revealed a more straight-forward interaction between PP2A and PKB. In 1996 Andjelković and colleagues reported that both vanadate and okadaic acid treatment of Swiss 3T3 cells led to an increased phosphorylation of PKB/Akt. This study implicated both a tyrosine phosphatase as well as PP1 or PP2A in the regulation of this kinase. The link between PKB/Akt and tyrosine phosphatases does not however lie in the direct phosphorylation of PKB/Akt, but rather in the upstream tyrosine kinase receptors involved in the activation of PKB/Akt (Bhuiyan & Fukinago, 2009). Andjelković *et al*. (1996) however also reported that incubation of PKB/Akt with PP2A led to the dephosphorylation of the kinase, thereby confirming PP2A as a phosphatase directly targeting PKB/Akt. Following these observations many others have also found that PP2A can dephosphorylate and thereby inactivate PKB/Akt, and as a result positively regulate downstream GSK-3β (which is inhibited when phosphorylated) in a wide variety of tissues (Salinas *et al.*, 2000; Resjö *et al*., 2001; Ivaska *et al*., 2002; Van Kanegan *et al.*, 2005; Mott *et al.*,

2008). This interaction between PP2A and PKB/Akt has also been shown in mouse neonatal cardiomyocytes, under the regulation of p38 MAPK (Zuluaga *et al.*, 2006).

Interestingly Andrabi *et al.* (2007) found that small T expression combined with growth factor treatment in NIH 3T3 cells led to an unbalanced phosphorylation of PKB/Akt between its two phospho residues (serine 473 and threonine 308) which actually favoured apoptosis. It therefore seems that under stimulatory conditions PP2A (or at least the specific holoenzyme affected by the presence of small T) only dephosphorylates threonine 308.

Since the phosphorylation of GSK-3β is tightly associated with PKB/Akt activity, not many studies have investigated the possibility of a direct interaction between PP2A and GSK-3β. In 1994 Sutherland & Cohen showed that phosphorylated, and therefore inactivated, GSK-3α and GSK-3β purified from rabbit skeletal muscle can be dephosphorylated and reactivated by the addition of PP2A. More recently Mitra and colleagues (2011) also showed a direct interaction between PP2A and GSK-3β in a melanoma cell line, where a heatshock protein, DNAJB6, can mediate the formation of a HSPA8 (heat-shock cognate protein, HSC70)-PP2A-GSK-3β complex.

Yao *et al.* (2011 & 2012) made some very interesting observations regarding the effects of GSK-3β on PP2A. In their 2011 publication they propose a mechanism by which activated GSK-3β mediates the inhibition of PTP-1B, thereby favouring the inhibitory phosphorylation of PP2A-C. At the same time GSK-3β can also reduce the expression of PP2A-C in a CREB (cAMP-response-element-binding protein) dependent manner. Their model therefore places GSK-3β as a negative regulator of PP2A, at least in human embryonic kidney-293 and mouse neuroblastoma (N2a) cells. Continuing with this focus, this group also reported that GSK-3β induces demethylation of PP2A-C, by increasing the expression of PME-1 and decreasing the levels of LCMT-1, also in human embryonic kidney-293 and mouse neuroblastoma cells (Yao *et* al., 2012).

PP2A has therefore been implicated in the negative regulation of the PKB/Akt – GSK-3β pathway. The relatively recent work by Yao *et al.* (2011 and 2012) however also reveals the possibility that GSK-3β can modulate PP2A, at least in human embryonic kidney-293 and mouse neuroblastoma cells. If one keeps in mind that inhibition of PKB/Akt (possibly by PP2A) can favour the activation of GSK-3β (possibly also by PP2A), one can speculate that GSK-3β-mediated inhibition of PP2A could be a negative feedback to keep GSK-3β activity in check. The effects of PP2A on PKB/Akt – GSK-3β has however received vey little attention in the context of the heart.

Summary

The potential interactions between PP2A and p38 MAPK, ERK p42/p44 and PKB/Akt – GSK-3β are shown in Figure 4.1. There is still much uncertainty concerning the interactions between PP2A and these signalling molecules; reflecting the complexity of these signalling pathways.

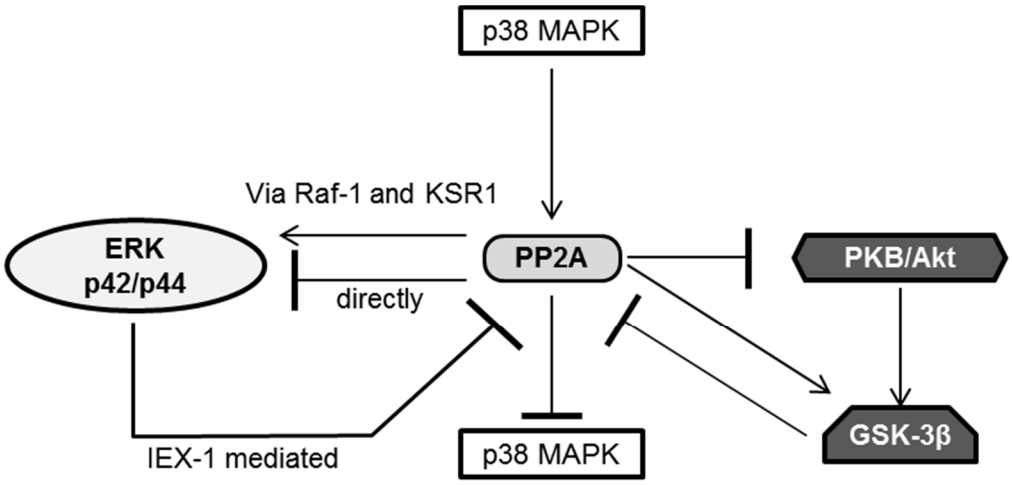


Figure 4.1. The possible interactions between PP2A and p38 MAPK, ERK p42/p44 and the PKB/Akt – GSK-3β pathway. p38 MAPK has been implicated as both an upstream activator as well as a downstream target of PP2A. ERK p42/p44 has been shown to be directly inhibited by PP2A, although PP2A-mediated dephosphorylation of inhibitory sites in activating proteins upstream of ERK p42/p44 place PP2A as a positive regulator of this pathway. ERK p42/p44 can also phosphorylate the B56 subunit of PP2A, thereby inhibiting the enzyme, however this effect seems to be limited to situations where both PP2A and ERK p42/p44 are bound to IEX-1. PP2A has also been identified as a negative regulator of PKB/Akt, as well as an activator of GSK-3β, either directly or via its effect on PKB/Akt. Intriguingly, GSK-3β has also been implicated as a negative regulator of PP2A.

Inhibition of PP2A as a tool to investigate its importance

The pharmacological modification of PP2A activity has been a dominant approach in investigating the contribution of PP2A to cellular events and signalling networks. It is especially its inhibition which has been pursued by many researchers, possibly simply due to the fact that there are very few direct activators of the enzyme.

PP2A inhibition: okadaic acid (OA)

For this study we utilized okadaic acid (OA) as a pharmacological tool to inhibit PP2A. OA is a marine toxin which was first decribed in the late 1970's and early 1980's as the active component responsible for diarrhetic shellfish poisoning in humans (Dounay & Forsyth, 2002; Dawson & Holmes, 1999). Although it was originally isolated from the black sea sponge *Halichondria okadaii*, it is actually synthesized by dinoflagellates (unicellular marine microorganisms) which are fed upon by marine sponges (Cohen *et al.*, 1990; Dounay & Forsyth, 2002). Since its isolation and

characterization, it has become one of the most used natural marine products in biological research in the world (Dounay & Forsyth, 2002). The reason for all this scientific interest in OA is due to its potent protein phosphatase inhibition capabilities.

Numerous studies have shown that OA is an inhibitor of the phosphoprotein phosphatases (PPPs) PP1 and PP2A, as well as the less abundant protein phosphatases 3, 4, 5 and 6 while only exerting an effect on other protein phosphatases at much higher concentrations or not at all (Cohen *et al.*, 1990; Honkanan *et al*., 1994; Herzig & Neumann, 2000; Janssens & Goris, 2001; Dounay & Forsyth, 2002). It is especially specific for PP2A, presenting with a 10-100 times higher IC_{50} value for PP2A than PP1. In this regard OA inhibits PP2A with an IC_{50} value of 0.2-1 nM, while the IC_{50} value for PP1 is in the range of 10-100 nM (Bialojan & Takai, 1988; Cohen *et al.*, 1990; Dawson & Holmes, 1999; Herzig & Neumann, 2000). As reported by Honkanan *et al*. (1994) OA also exhibits a lower IC_{50} value for PP3 than PP1, but still the lowest for PP2A (IC_{50} values (in nM): PP2A: 0.28; PP3: 3.91; PP1: 49.0). An explanation for this specificity for PP2A was recently put forward by Xing and colleagues (2006) who found that OA binds to an area located right above the active site of PP2A-C with a hydrophobic cage, which is absent in PP1, accomodating the hydrophobic end of OA.

Early kinetic studies (Bialojan & Takai, 1988) showed that OA acts as a reversible, non-competitive or mixed inhibitor of its target phosphatases. Although its interaction with PP2A is reversible, it is still very tight, as demonstrated by Favre and colleagues (1997) who reported that three to four ethanol precipitations were required to reactivate OA inhibited PP2A, in comparison to a single ethanol precipitation which could induce the complete dissociation of the regulatory subunits from PP2A-C.

OA is a hydrophobic polyether fatty acid which therefore has the ability to transverse cell membranes, making it a very useful tool for the study of cellular PP2A and PP1 (Cohen *et al.*, 1990; Dawson & Holmes, 1999; Dounay & Forsyth, 2002). That being said, OA has some crucial limitations which must be kept in mind. Although it can cross cell membranes, it does so with some difficulty (Herzig & Neumann, 2000). That, combined with the fact that PP1 and PP2A are often present at quite high levels in some cell types (0.1-1.0 µM) (Cohen *et al.*, 1990) necessitate the use of higher concentrations of OA than the IC_{50} values generated under *in vitro* conditions. The consequence of this is that there could be a degree of uncertainty regarding the precise phosphatase (PP1 or PP2A) being inhibited by a given concentration of OA.

Keeping in mind the major contribution of PP1 and PP2A to protein dephosphorylation in mammalian cells, it is no surprise that administration of OA can induce a major increase in cellular protein phosphorylation, even within minutes (Cohen *et al.*, 1990). In isolated guinea pig ventricular

cardiomyocytes, Neumann *et al.* (1993) found that a 5 minute incubation with 1, 10 and 30 µM of OA induced an increase in phosphorylation of several proteins, with maximum phosphorylation elicited by 10 µM. This however presents with a new problem: OA treatment may elicit a pleiotrophic multitude of effects, making the interpretation of data even more difficult (Dawson & Holmes, 1999).

The wide array of cellullar responses associated with OA treatment is reflected by the various effects which OA exerts in different tissues. OA has been associated with tumor progression, but has also been characterized as a tumor suppressor, it induces cytotoxicity and apoptosis, it is a neurotoxin and elicits a diarrhoeagenic effect (Cohen *et al.*, 1990; Jayaraj *et al.*, 2008; Munday, 2013). The extent to which phosphatase inhibition contributes to some of its toxic effects is however debatable (Munday, 2013).

In summary, OA is a work horse for the study of the protein phosphatases PP1 and PP2A. Due to its preferential inhibition of PP2A at considerably lower concentrations than PP1, OA can be used to specifically target PP2A. Its use and data generated by its administration must however be approached with caution, since its relatively low cell membrane permeability necessitates the use of OA at higher dosages than would be ideal.

These limitations are however also present with other inhibitors of PP1 and PP2A and are indeed characteristic of the intrinsic difficulties associated with investigating the protein phosphatases.

Motivation, hypothesis and aims

It is notable in the literature that very little research has been done concerning the interactions between PP2A and p38 MAPK, ERK p42/p44 and PKB/Akt – GSK-3β in the heart, and specifically in the setting of I/R injury. Work in other cell systems and tissues however provide compelling evidence that PP2A could be a regulator of these pathways in the heart. This possibility must be investigated in view of the proposed importance of the protein phosphatases in regulating cellular signalling (Heinrich *et al.*, 2002 & Hornberg *et al.*, 2005) and the continuous requirement for more knowledge and a better understanding of the signalling events associated with myocardial I/R in order to advance the efficacy of cardioprotective interventions.

The purpose of this part of the project was therefore to investigate the importance of PP2A in myocardial I/R injury and to better define the participation of PP2A within the signalling networks of p38 MAPK and the RISK pathways in this setting.

In order to achieve this purpose we set about accomplishing the following aims:

1.) Determining the consequence of PP2A inhibition in conjunction with I/R in an isolated working rat heart model on infarct size, as well as post-ischaemic function.
2.) Investigating the mechanism by which PP2A inhibition elicited its effects in the heart by focusing on the expression and phosphorylation of p38 MAPK, ERK p42/p44, PKB/Akt, GSK-3β, as well as PP2A-A, PP2A-C and the methylation and phosphorylation of PP2A-C.

As has been reported by several others (Armstrong *et al.*, 1997; Isotani *et al.*, 2002; Sariahmetoglu *et al*., 2012), we expect to find that PP2A inhibition will be cardioprotective due to an increased phosphorylation and activation of pro-survival elements – specifically ERK p42/p44 and PKB/Akt.

Concerning the signalling dynamics we hypothesise that phosphorylation of p38 MAPK during sustained ischaemia is an upstream event from PP2A. PP2A will however probably be involved in the dephosphorylation of both ERK p42/p44 and PKB/Akt during ischaemia. The dephosphorylation of PKB/Akt will favour GSK-3β dephosphorylation and activation during ischaemia, a process to which PP2A might contribute directly. During initial reperfusion the reduction in PP2A activity (Chapter 3) will present an opportunity for the phosphorylation and activation of the pro-survival pathways.Materials and Methods

Material and Methods

Rationale

In order to shed more light on the importance and role of PP2A in myocardial I/R injury we followed an approach based on the administration of pharmacological modulators of PP2A activity to hearts exposed to I/R whereafter infarct size, functional recovery and kinase profiles were assessed (Figure 4.2).

This chapter will specifically focus on the effects of OA mediated inhibition of PP2A within this setting, while chapter 5 will focus on the effects of PP2A activation.

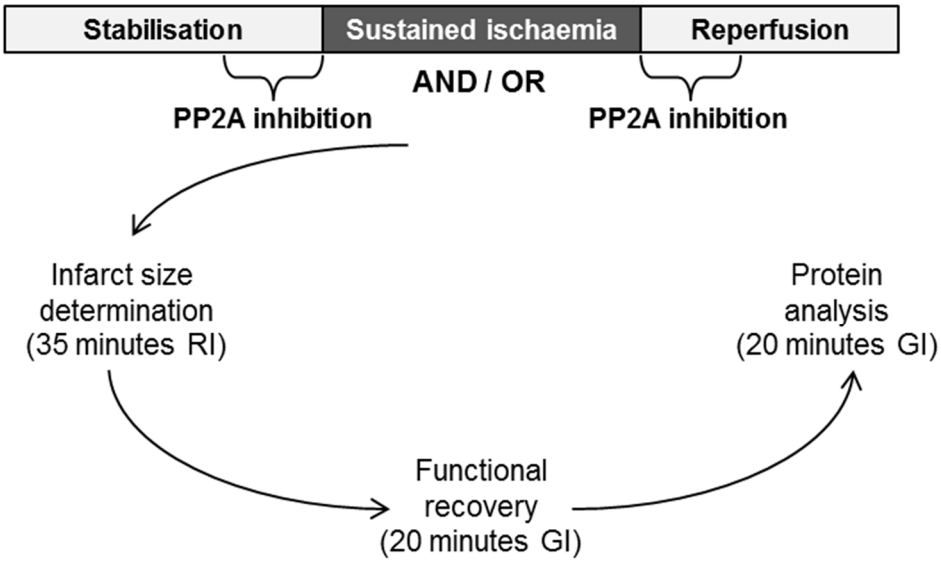


Figure 4.2. Schematic presentation of the approach followed to investigate the effects of PP2A inhibition on the outcomes and signalling pathways associated with myocardial I/R.
An isolated working rat heart exposed to sustained ischaemia in conjunction with PP2A inhibition was used. We first determined the effects of PP2A inhibition on infarct size in a model of 35 minutes regional ischaemia (RI). The PP2A inhibitor administration protocol which elicited some kind of signifcant response (positive or negative) was then further investigated in terms of its effects on functional recovery following 20 minutes of global ischaemia (GI), as well as the protein profiles of PP2A and the RISK and MAPK pathways.

Protocols

OA dosage and administration

Okadaic acid was obtained from Calbiochem as okadaic acid sodium salt (# 459620). For all experiments we utilized OA at a concentration of 10 nM. We selected this concentration because: (1.) It falls safely within the IC_{50} value range for the OA mediated inhibition of PP2A, without coming close to the IC_{50} range for PP1. Since our focus is PP2A, it was obviously very important to try and inhibit only PP2A. (2.) Similar dosages have been reported in the literature. A slightly lower concentration of 7.5 nM has been used in our laboratory before (Fan *et al.*, 2010). Ladilov *et al.* (2002) also found that 5 nM of OA partially enhanced the protection afforded by hypoxic preconditioning in an isolated rat heart model.

As shown in Figure 4.2 we tested the effects of PP2A inhibition on infarct size development by administering OA either directly before sustained ischaemia and/or at the very onset of reperfusion. Which ever of these protocols elicited a significant effect on infarct size was then further investigated to assess its effects on functional recovery and determine the phosphorylation profiles of the RISK pathways as well as p38 MAPK.

Infarct size (IFS)

The PP2A inhibitor OA was administered at a PP2A specific concentration of 10 nM for a period of 10 minutes directly prior to 35 minutes of sustained regional ischaemia (RI) and/or at the onset of reperfusion (Figure 4.3). Reperfusion administration was accomplished by administering OA for the final 5 minutes of RI, as well as the first 10 minutes of reperfusion. As described in Chapter 2, during ischaemia hearts were monitored and the temperature maintained at 36.5 ºC. Hearts were reperfused for a total of 60 minutes, whereafter the left descending coronary artery was permanently occluded and the viable area stained with 0.5% Evans Blue dye. Hearts were then frozen and later stained with TTC to further delineate the necrotic infarcted zone and the area at risk (AAR), defined as viable tissue in the area which did not recieve perfusion during the RI intervention.

Many workers in the field prefer to expose the hearts to longer periods of reperfusion (up to 120 or 180 minutes) to ensure complete formation and stabilisation of the infarct before measuring its size. We however opted for a shorter period of time, since previous work in our laboratory has shown that shorter reperfusion periods do not influence the relative degree of IFS development between groups (Marais *et al*., 2005; Fan *et al.*, 2009).

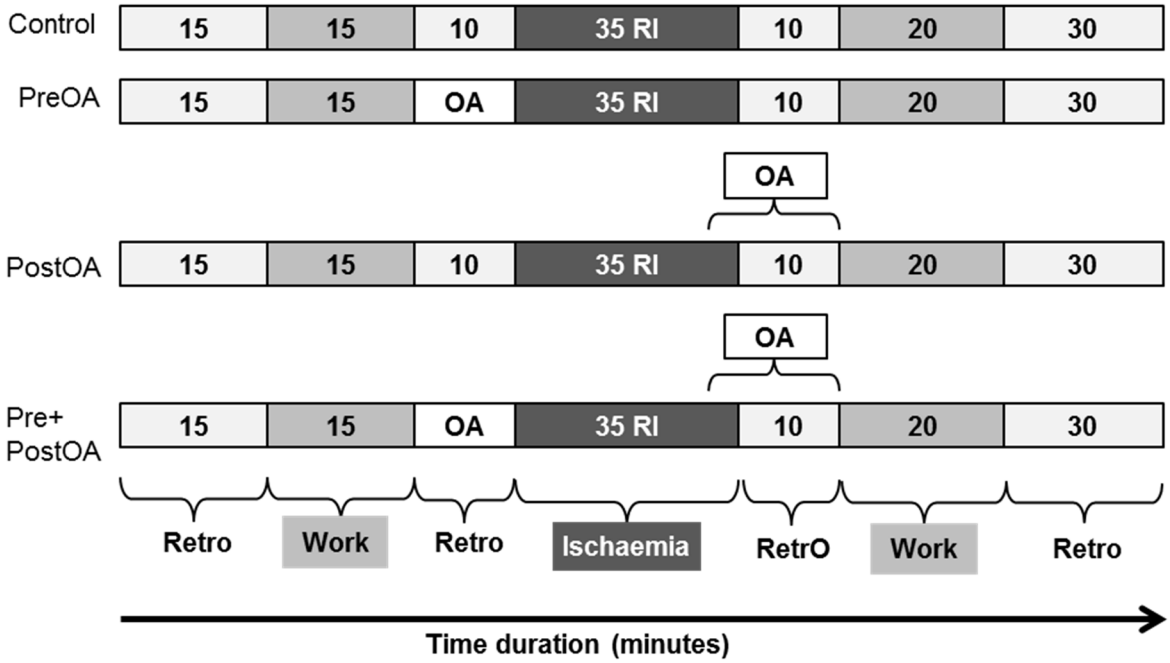


Figure 4.3. Protocols for determining the effects of okadaic acid (OA; 10 nM) mediated PP2A inhibition on infarct size.
Three administration protocols were followed: pretreatment for 10 minutes directly before regional ishcaemia (RI); treatment at the very onset of reperfusion (PostOA, for the final 5 minutes of RI and the first 10 minutes of reperfusion) and a combination of these two protocols (Pre+PostOA).

Functional recovery

We found that pretreatment with OA (PreOA) was the only protocol which elicited a signifcant effect on IFS. We therefore focussed our attention on the PreOA protocol to determine if it could also influence functional recovery following 20 minutes of global ischaemia (GI) (Figure 4.4 A).

Protein profiles

Using standard Western blotting techniques we also sought to determine the effects of PreOA treatment on the expression and phosphorylation of PP2A-A, PP2A-C, phosphorylated PP2A-C and nonmethylated PP2A-C. In order to shed light on the mechanisms by which PreOA treatment elicits its effect, we also determined the expression and phosphorylation of PKB/Akt, GSK-3β, ERK p42/p44 and p38 MAPK. For all these experiments we utilized 20 minutes GI in order to generate homogenous tissue for analysis. Since OA was administered before sustained ischaemia we had to determine its effects both at the end of sustained ischaemia, as well as at the onset of reperfusion (Figure 4.4 B). As motivated elsewhere we were specifically interested in the clinically relevant first moments of reperfusion, we therefore collected tissue at both 5 and 10 minutes reperfusion.

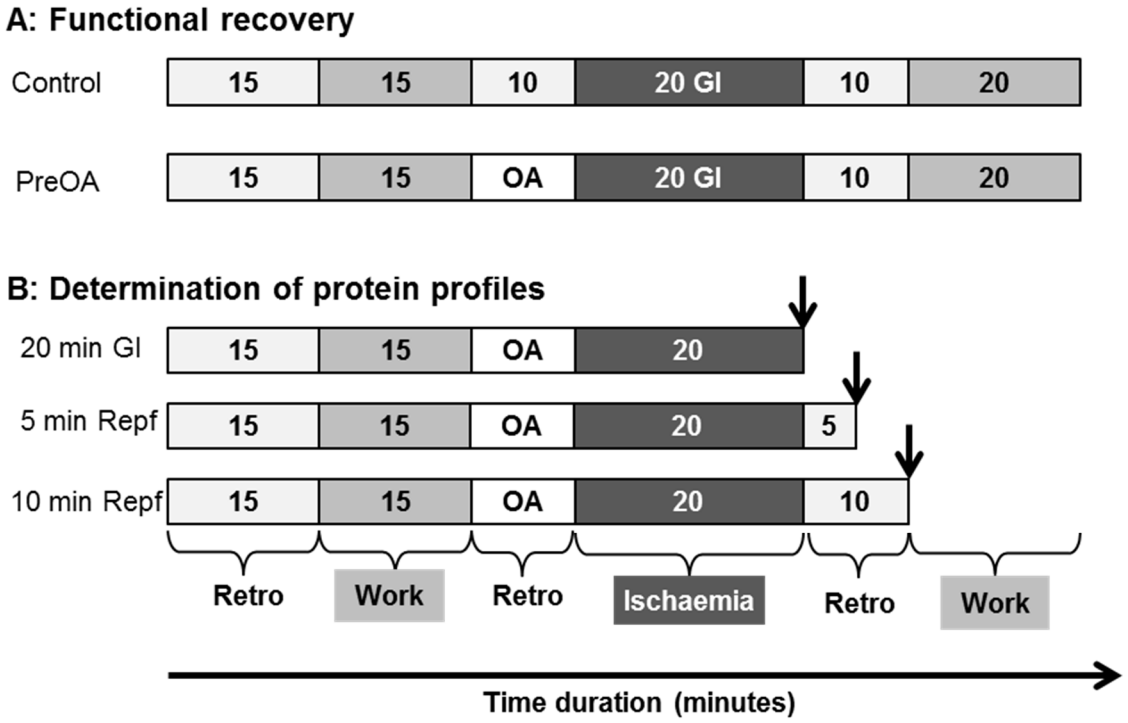


Figure 4.4. Investigating the effects of pretreatment with OA on functional recovery and protein profiles.
(A) Hearts were exposed to 10 minutes of OA (10 nM) directly prior to 20 minutes of global ischaemia and functional recovery following 30 minutes reperfusion determined as a percentage of baseline function. (B) Samples were also collected at the end of ischaemia, as well as after 5 and 10 minutes reperfusion, for the determination of the protein profiles of PP2A, PKB/Akt, GSK-3β, ERK p42/p44 and p38 MAPK. For each timepoint investigated OA administration was compared to an appropriate control group which was exposed to a similar protocol, but in the absence of OA administration.

Combined with these Western blotting experiments, we also performed immunoprecipitation (as described in Chapter 2) of PP2A-C at 5 minutes reperfusion in an attempt to confirm its association with the RISK and MAPK pathways under investigation.

Statistical analysis

Unpaired Student T-tests were utilized in all cases where only two groups were compared. For the analysis of more than two groups an ANOVA was performed. For data shown in the graphs all groups were compared with each other by applying the Bonferroni *post hoc* test. Whenever multiple experimental groups were compared with a single common control group alone, Dunnett's *post hoc* test was additionally performed. A P-value of less than 0.05 was deemed significant.

Results

The effect of okadaic acid mediated PP2A inhibition on infarct size

In order to better define the contribution of PP2A to the development of I/R injury we inhibited PP2A in an isolated working rat heart model exposed to 35 minutes of regional ischaemia with infarct size as primary endpoint. As discussed in Chapter 1, ischaemia and reperfusion, although linked, can be seen as two separate molecular entities. For this reason we administered OA either before sustained ischaemia, or during the onset of reperfusion. We limited OA administration to the first 10 minutes of reperfusion, since the existence of early-reperfusion interventions such as PostC identified early reperfusion as an important period in the development of reperfusion injury. A group was also included in which OA was administered both before ischaemia, as well as during early reperfusion (Figure 4.3). For these experiments we utilized rudimentary measurements to assess the quality and function of the isolated hearts at the end of the stabilisation period (table 4.1): coronary flow (CF) during retrograde perfusion, and during work mode aortic output (AO) and cardiac output (CO). There were no signifcant differences between any of the groups with regards to any of these parameters.

Table 4.1. Baseline functional parameters of isolated rat hearts subsequently exposed to 35 minutes of RI in conjunction with okadaic acid administration.
† Coronary flow as measured during normal retrograde perfusion.

Group	Coronary flow† (ml/min)	Aortic output (ml/min)	Cardiac output (ml/min)	n-value
Control	8.25±0.29	46.56±2.50	62.81±3.02	16
PreOA	7.69±0.21	49.00±1.96	66.25±2.82	8
PostOA	7.79±0.59	45.53±2.20	60.53±2.97	7
Pre+PostOA	9.08±0.52	52.50±1.54	68.58±1.89	6

Following OA administration and exposure to 35 minutes of RI, hearts were reperfused for a total of 60 minutes before staining and quantification of infarct size (Figure 4.5). Of the three OA administration protocols which were followed, it is only pretreatment with OA which induced a signifcant change in the percentage infarct size by reducing it (Control: 41.53±2.81% vs PreOA: 26.26±5.10%, n=8-16; P<0.05). This reduction in infarct size was independent of any changes in the area at risk.

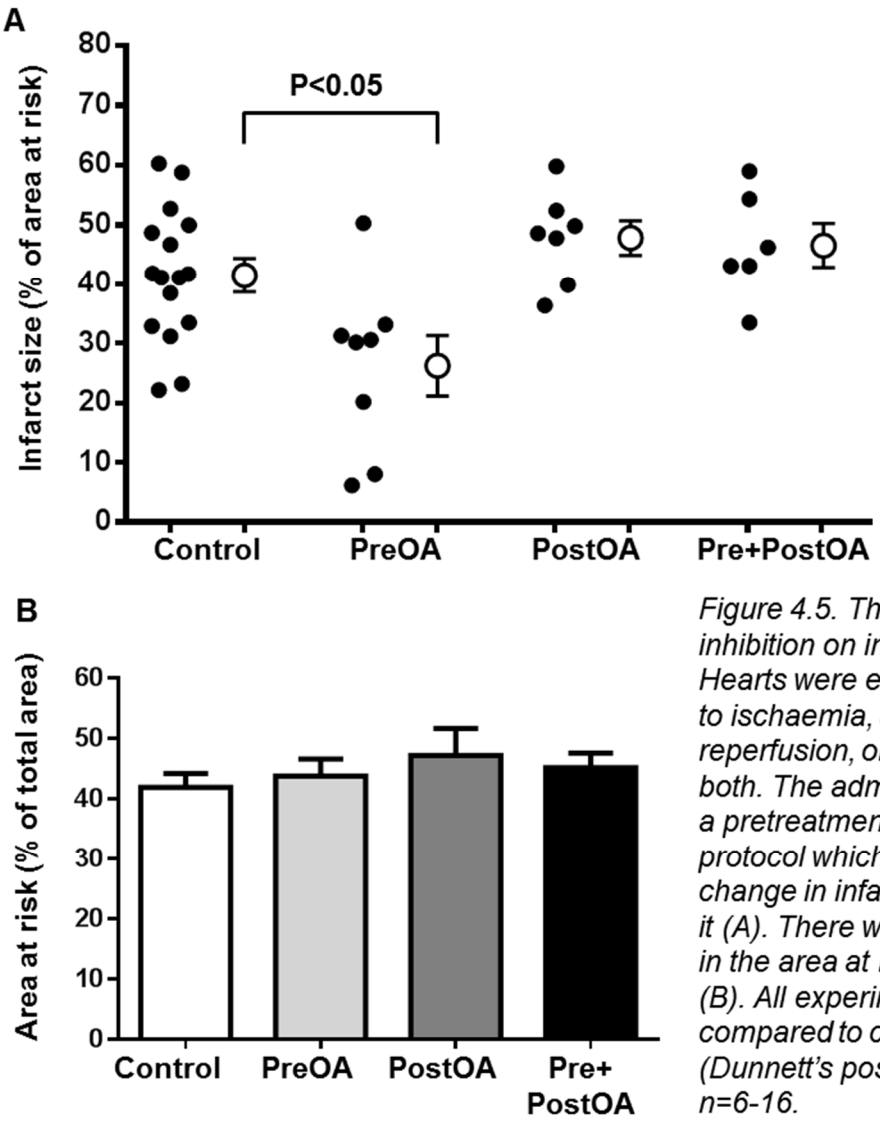


Figure 4.5. The effect of PP2A inhibition on infarct size. Hearts were exposed to OA prior to ischaemia, at the onset of reperfusion, or a combination of both. The administration of OA as a pretreatment was the only protocol which elicited a signifcant change in infarct size by reducing it (A). There were no differences in the area at risk between groups (B). All experimental groups were compared to control alone (Dunnett's post hoc test). n=6-16.

Midway through the reperfusion period we also assessed the functional recovery of the hearts by measuring AO and CO at the end of the reperfusion work mode and expressing these parameters as a percentage of the pre-ischaemic baseline values (table 4.2). OA treatment failed to exert any effect in terms of functional recovery, even the infarct sparing PreOA treatment was associated with very poor functional recovery (AO: Control: 27.00±4.09% vs. PreOA: 19.00±6.42%; CO: Control: 48.94±4.60% vs. PreOA: 36.25±5.79%).

Table 4.2. Functional recovery of hearts treated with OA and exposed to 35 minutes of RI. None of the OA treatment protocols was associated with significant changes in post-ischaemic functional ability.

Group	Recovery as a percentage of baseline		n-value
	Aortic output	Cardiac output	
Control	27.00±4.09	48.94±4.60	16
PreOA	19.00±6.42	36.25±5.79	8
PostOA	18.57±6.59	37.29±5.61	7
Pre+PostOA	25.33±5.86	41.50±4.96	6

These results identified pretreatment with OA prior to sustained ischaemia as an infarct sparing intervention, although it failed to improve functional recovery in this model of ischaemia.

The effect of OA administration prior to ischaemia on functional recovery in a model of global ischaemia

Having established that pretreatment with OA exerted an infarct sparing effect, we wanted to investigate the signalling events associated with this protection. To do this we wanted a homogeneous tissue sample for Western blotting analysis, in contrast to the heterogenous cardiac preparation generated by regional ischaemia where per definition only a portion of the heart is exposed to I/R, while the rest (viable area (VA)) receives normal perfusion throughout. This left us with two options: either dissect the AAR from a heart exposed to RI and analyse that, or expose the whole heart to ischaemia (GI). Dissection of the AAR however has two drawbacks: (1.) it yields only a small amount of tissue for analysis; and (2.) there is the risk of accidentaly including VA in the sample. We therefore opted for the latter option of exposing the hearts to GI. This approach however had the major disadvantage that it necessitated the characterization of a new perfusion protocol. We therefore had to assess the effects of pretreatment with OA on functional recovery in this new model.

For these experiments hearts were exposed to 20 minutes of GI followed by 30 minutes of reperfusion (Figure 4.4 A). Since function was the primary endpoint for these experiments we also measured work performance in conjunction with AO and CO (see Chapter 2). Baseline stabilisation values for the hearts used are shown in table 4.3.

*Table 4.3. Baseline functional parameters of hearts exposed to 20 minutes of GI in the presence or absence of OA administration for a period of 10 minutes prior to sustained ischaemia. *mW: milliWatt.*
† Coronary flow as measured during normal retrograde perfusion.

Group	Coronary flow (ml/min) †	Aortic output (ml/min)	Cardiac output (ml/min)	Total work (mW*)	n-value
Control	8.33±0.26	48.00±2.51	64.44±2.53	14.44±0.47	9
PreOA	8.78±0.29	49.89±1.69	67.22±1.96	15.12±0.75	8

At the end of reperfusion, function was measured and expressed as a percentage of pre-ischaemic function, thereby indicating the degree of functional recovery. In contrast to the infarct sparing

effects observed after 35 minutes of RI, PreOA failed to staticitically increase functional recovery after 20 minutes GI (Figure 4.6).

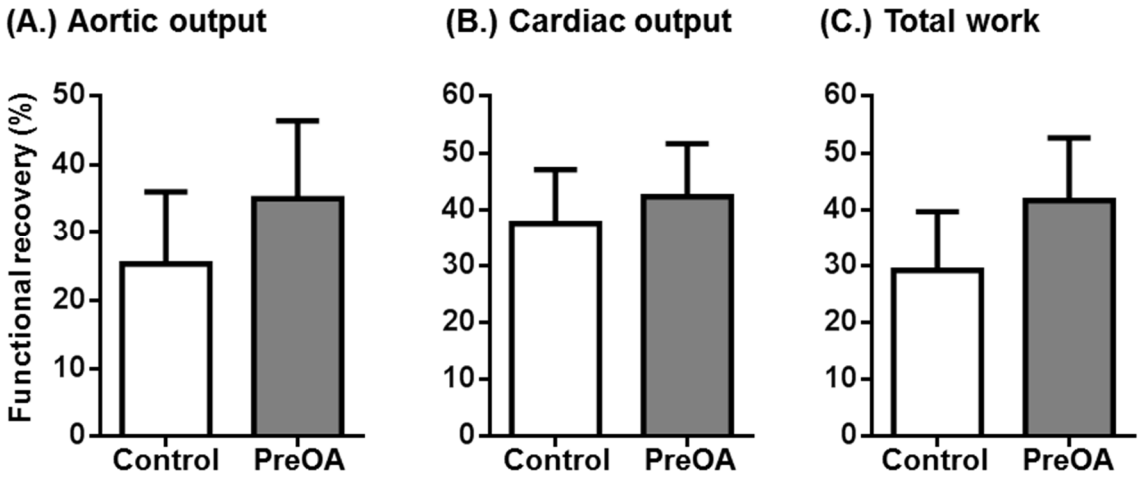


Figure 4.6. The effect of PP2A inhibition prior to ischaemia on functional recovery. OA was administered to hearts for a period of 10 minutes directly before 20 minutes of GI. Although OA treatment was associated with an increasing trend in the parameters measured, it never reached significance. Parameters measured were aortic output (A), cardiac output (B) and total work (C). Graphs show recovery defined as post-ischaemic function expressed as a percentage of baseline values. n=8-9.

Pre-ischaemic treatment with OA was therefore unable to elicit an increase in functional recovery following 20 minutes of GI.

Protein profiles elicited by OA treatment prior to sustained ischaemia followed by reperfusion

Although the administration of OA prior to 20 minutes GI failed to exert a significant cardioprotective effect, in contrast to its infarct sparing effects in a model of 35 minutes RI, it could still be used as a model of OA treatment in the context of ischaemia and reperfusion *per se*. We therefore collected samples at the end of 20 minutes GI, as well as at 5 and 10 minutes reperfusion, and analysed the expression and phosphorylation of PP2A, PKB/Akt, GSK-3β, p38 MAPK and ERK p42/p44 following pre-ischaemic treatment with OA in an attempt to shed light on the infarct sparing effects of PP2A inhibition (Figure 4.4 B).

Protein profiles after 20 minutes of GI

Protein phosphatase 2A

Twenty minutes of ischaemia was not associated with any changes in the expression of PP2A-C or PP2A-A (Figure 4.7). This is in starck contrast to our earlier characterization of PP2A. In Chapter 3 we reported that 20 minutes of GI was associated with a significant increase in PP2A-C levels, combined with an upward shift in the ratio of PP2A-C to PP2A-A (Figure 3.18). This increase was however small and best seen with a high n-value, while for these experiments we only had four hearts in the control group. It must however be noted that these four controls were included in the analysis depicted in Figure 3.18 which confirmed the ischaemia-induced increase in PP2A-C levels.

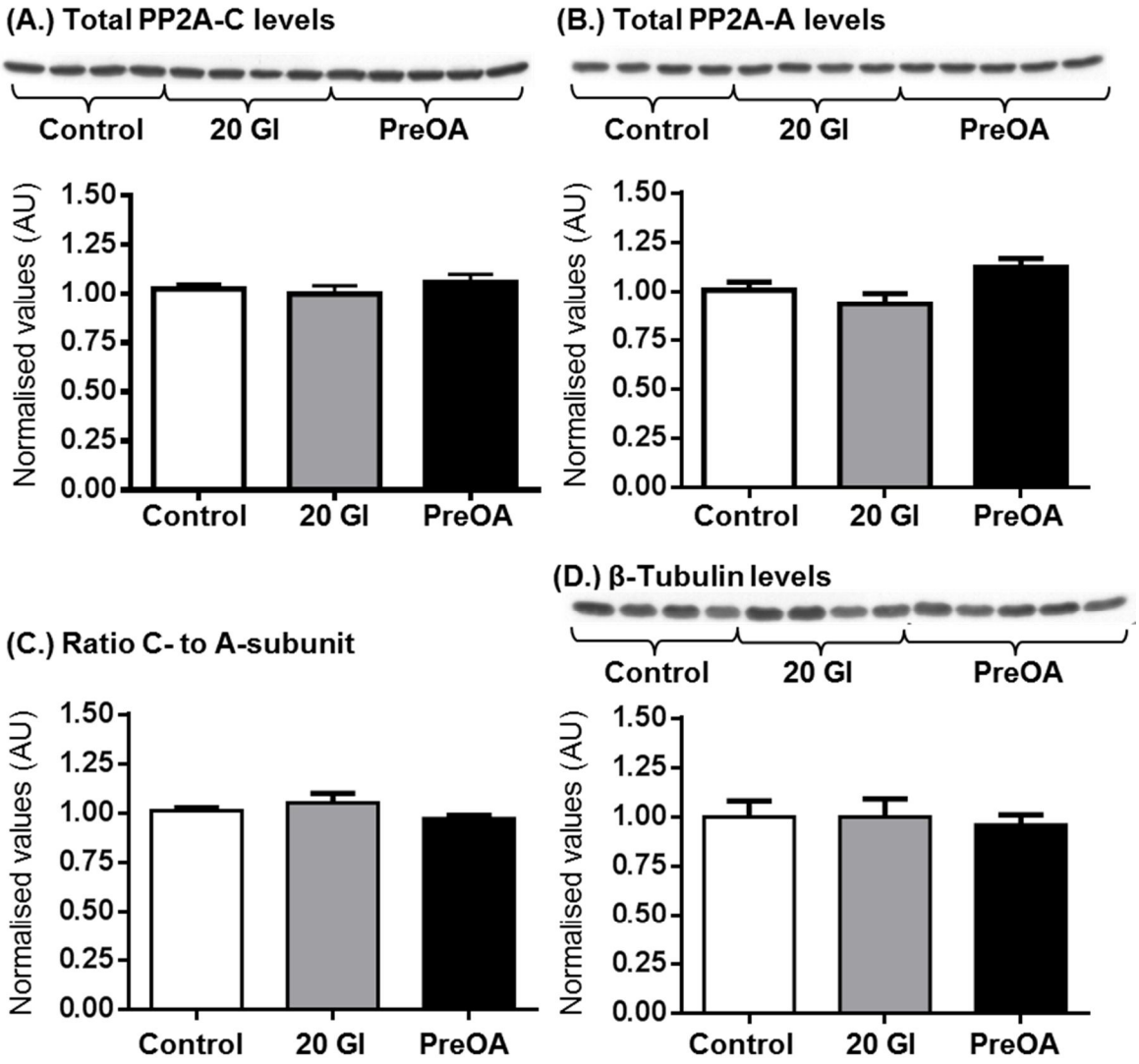


Figure 4.7. Expression of PP2A-A and PP2A-C at 20 minutes GI following pretreatment with OA.
OA failed to elicit any effect on the total protein levels of PP2A-C (A) and PP2A-A (B). β-Tubulin was utilized as a loading control. n=4-5.

Interestingly, as noted in Chapter 3, the anti-bodies against nonmethylated PP2A-C and phosphorylated PP2A-C sometimes detected a double band (Figure 4.8). This double band could be indicative of the two PP2A-C isoforms (PP2A-Cα and –β). We however did not distinguish between them but rather combined the two bands in a single analysis.

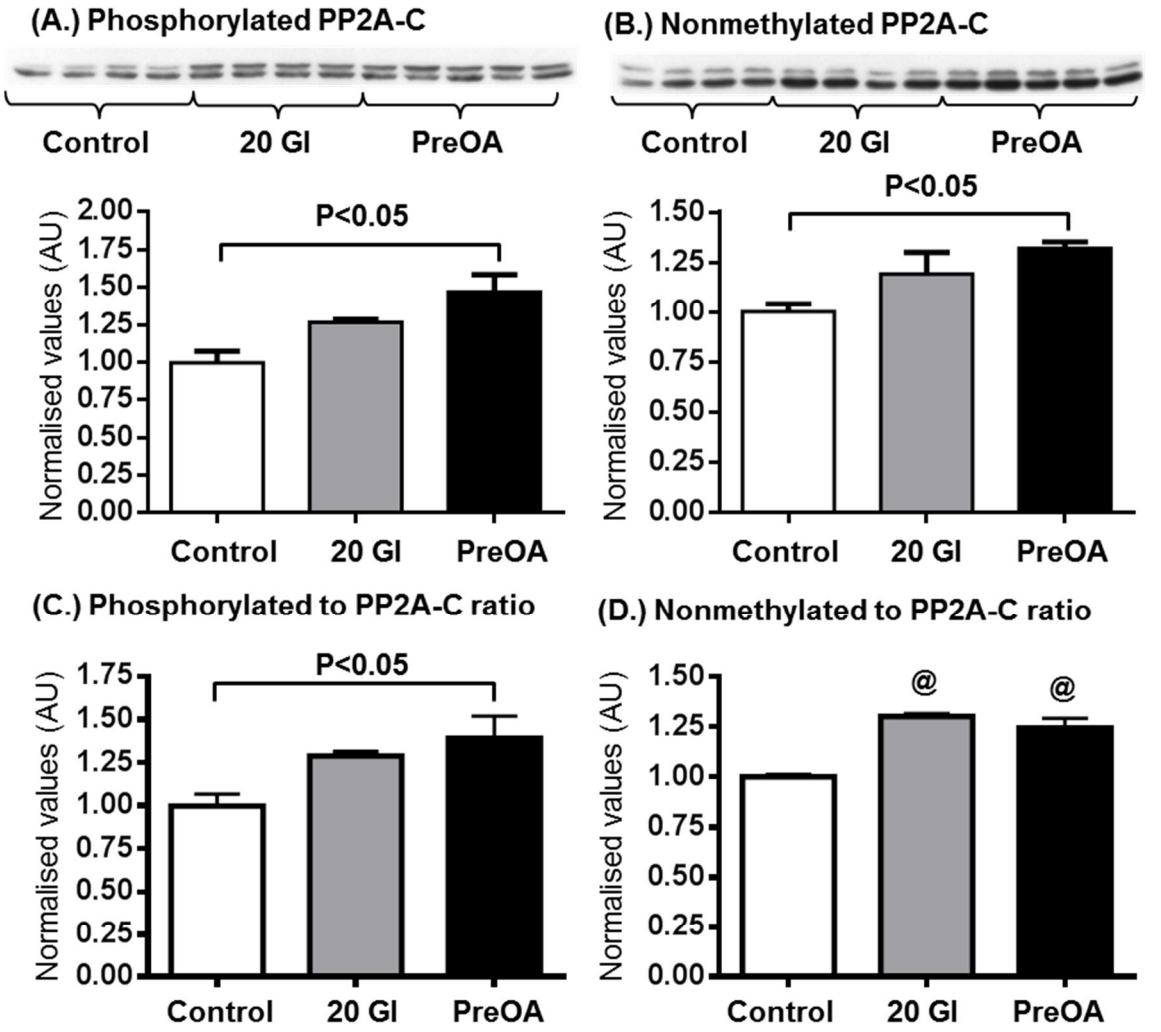


Figure 4.8. Phosphorylation and methylation of PP2A-C in hearts pretreated with OA at 20 minutes GI.
OA treatment was associated with an increase in phosphorylation (A&C), as well as demethylation (B & D) of PP2A-C relative to non-ischaemic control. n=3-5; @: P<0.05 vs Control.

OA treatment was associated with an increase in the phosphorylation of PP2A-C in comparison to a non-ischaemic control (Figure 4.8: Absolute phosphorylated PP2A-C: Control: 1.00±0.08 AU vs PreOA: 1.47±0.11 AU, n=4-5; P<0.05, and relative to total PP2A-C: Control: 1.00±0.07 AU vs PreOA: 1.39±0.13 AU, n=4-5; P<0.05). The absence of a signifcant increase in PP2A-C phosphorylation in the ischaemic group, in contrast to the data in Figure 3.18, can also be attributed to limited sample size. Absolute nonmethylation was also elevated in the OA treatment group in comparison to a non-ischaemic control (Figure 4.8: Control: 1.00±0.04 AU vs PreOA: 1.32±0.03 AU, n=3-5; P<0.05). The degree of demethylation of the PP2A-C population (Figure 4.8

D) was similar in the OA and ischaemic groups, while both were elavated compared to control (Control: 1.00±0.01 AU vs. 20 GI: 1.30±0.02 AU and PreOA: 1.24±0.05 AU, n=3-5; P<0.05).

Pretreatment with OA therefore elicited an increase in phosphorylation and reduction in methylation of PP2A-C as evidenced by the differences between the OA treated group and the non-ischeamic control.

Protein kinase B (Akt)

Sustained ischaemia was associated with a reduction in the phosphorylation of PKB/Akt at serine 473 compared to the positive control exposed to 20 minutes GI followed by 30 minutes reperfusion. The administration of OA had no effect on this profile (Figure 4.9).

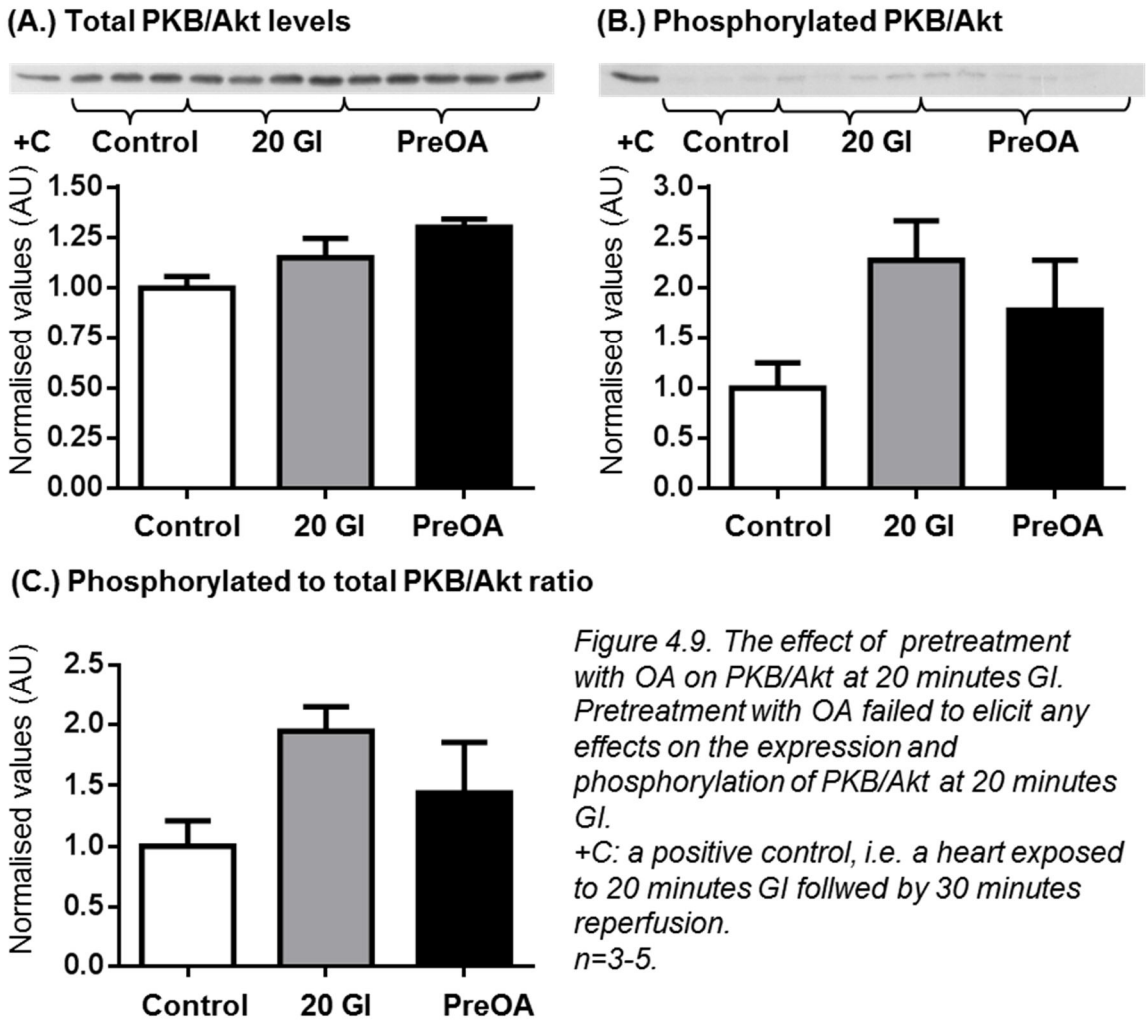


Figure 4.9. The effect of pretreatment with OA on PKB/Akt at 20 minutes GI. Pretreatment with OA failed to elicit any effects on the expression and phosphorylation of PKB/Akt at 20 minutes GI.
+C: a positive control, i.e. a heart exposed to 20 minutes GI follwed by 30 minutes reperfusion.
n=3-5.

Glycogen synthase kinase-3β

Pre-ischaemic treatment with OA was associated with an increase in the phosphorylation of GSK-3β relative to control values (Figure 4.10: Phosphorylated GSK-3β: Control: 1.00±0.21 AU vs. PreOA: 3.44±0.33 AU, n=3-5; P<0.05; and Phosphorylated to total ratio: Control: 1.00±0.17 AU vs. PreOA: 4.04±0.28 AU, n=3-5; P<0.05). Although ischaemia *per se* was not associated with a statistical elevation in phosphorylated GSK-3β this can be ascribed to the degree of variation in the data, as well as as the type of statistical analysis applied. The reason for the observed variation in the data is unknown, although it seems to indicate that GSK-3β phosphorylation is very sensitive to external stimuli and experimental conditions. Direct comparison of the ischaemic group with control, using an ANOVA followed by Dunnett's *post hoc* test, reveals a statistical difference (Phosphorylated to total GSK-3β ratio: Control: 1.00±0.17 AU vs. 20 GI: 3.43±0.97 AU, n=3-4; P<0.05 with Dunnett's correction).

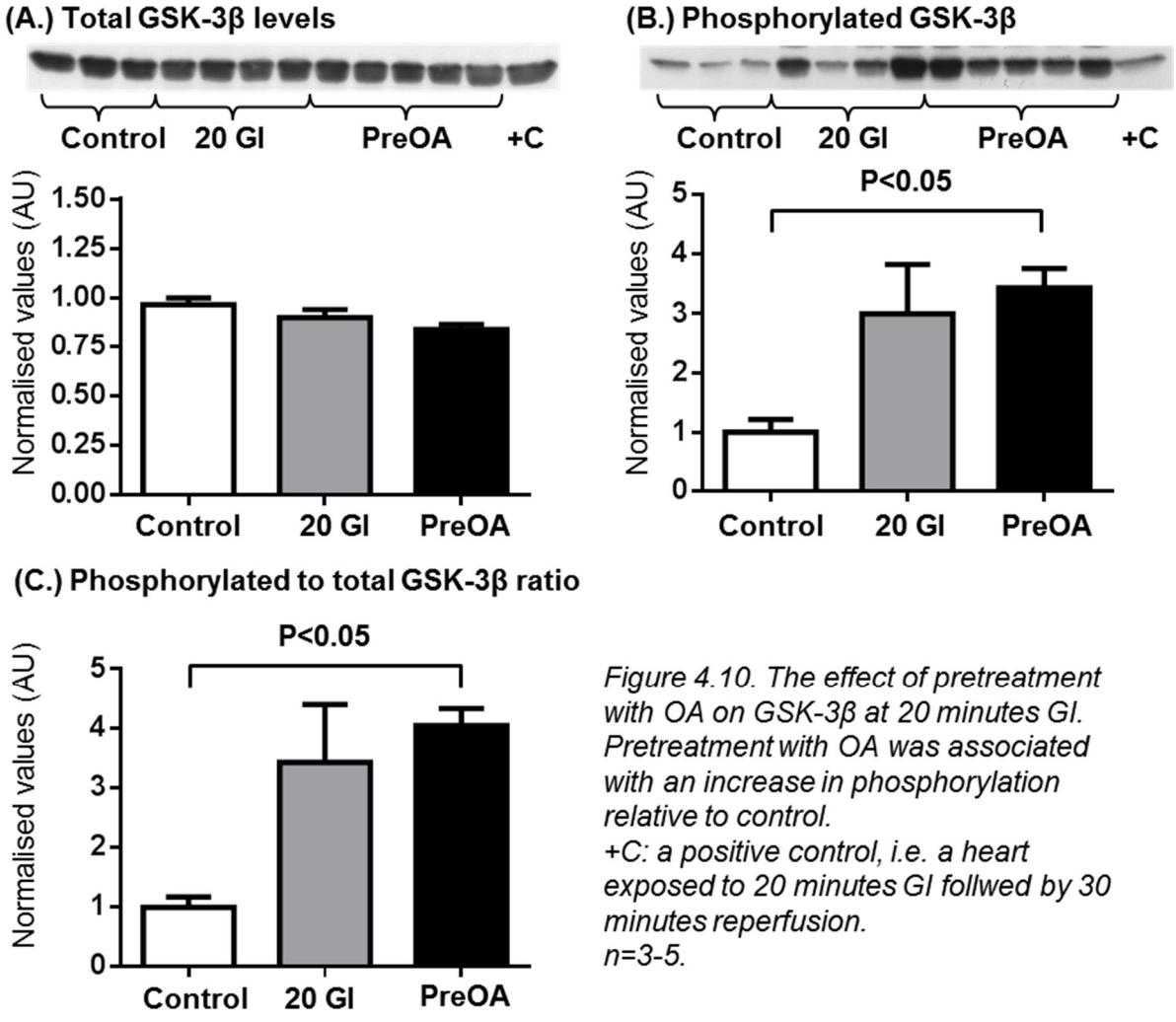


Figure 4.10. The effect of pretreatment with OA on GSK-3β at 20 minutes GI. Pretreatment with OA was associated with an increase in phosphorylation relative to control.
+C: a positive control, i.e. a heart exposed to 20 minutes GI follwed by 30 minutes reperfusion.
n=3-5.

Our data therefore reveals that ischaemia induces an increase in the phosphorylation of GSK-3β which is slighty enhanced by the administration of OA.

p38 Mitogen activated protein kinase

As was observed for GSK-3β, ischaemia induced the phosphorylation of p38 MAPK. In this instance we therefore expressed all data relative to 20 minutes GI and not control (Phosphorylated to total ratio: Control: 0.10±0.06 AU vs. 20 GI: 1.00±0.21 AU, n=3-4; P<0.05). Inhibition of PP2A was not associated with any changes in this ischaemia-induced phosphorylation of p38 MAPK (20 GI: 1.00±0.21 AU vs. PreOA: 1.14±0.13 AU, n=4-5) (Figure 4.11).

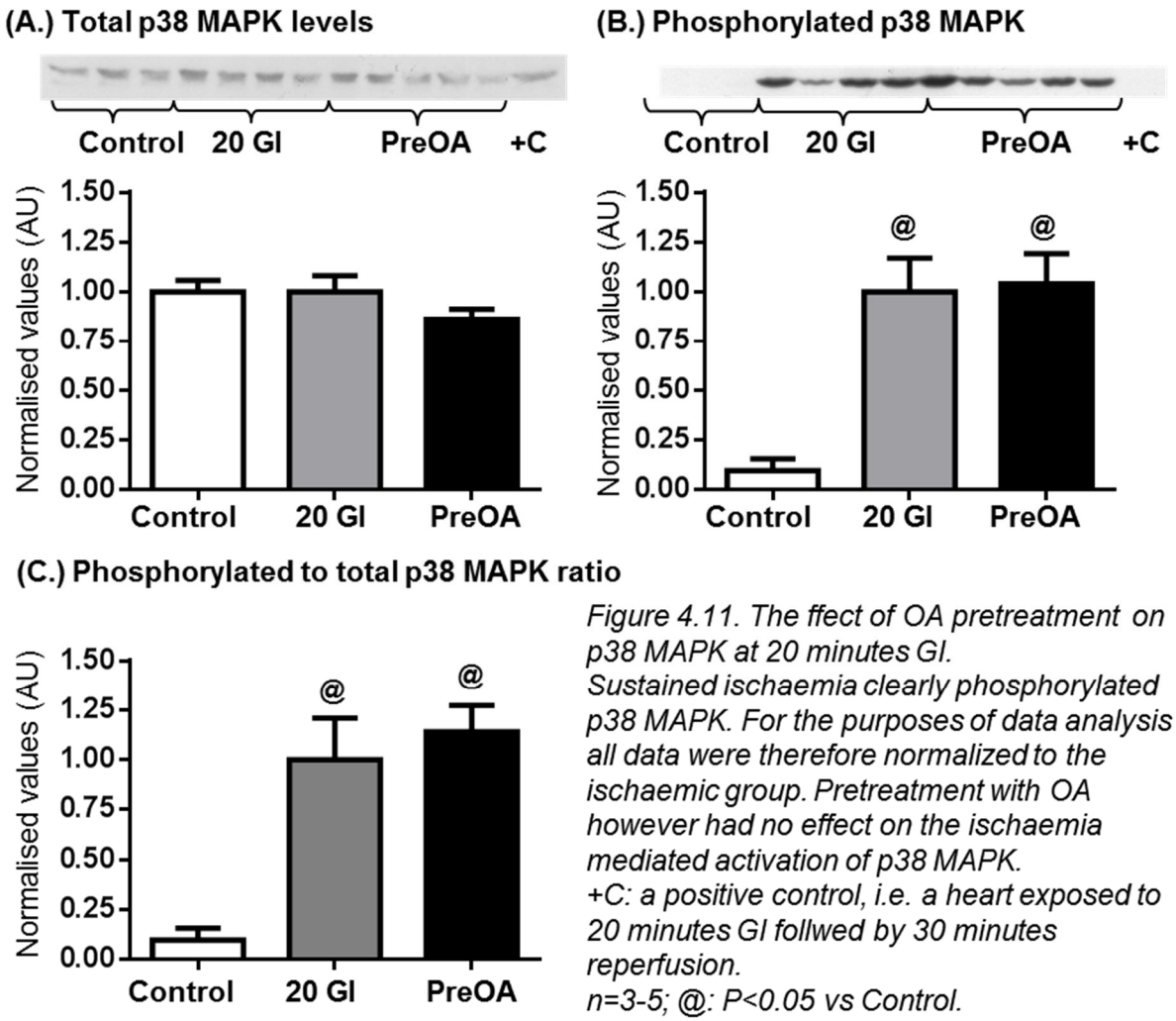


Figure 4.11. The ffect of OA pretreatment on p38 MAPK at 20 minutes GI.
Sustained ischaemia clearly phosphorylated p38 MAPK. For the purposes of data analysis all data were therefore normalized to the ischaemic group. Pretreatment with OA however had no effect on the ischaemia mediated activation of p38 MAPK.
+C: a positive control, i.e. a heart exposed to 20 minutes GI follwed by 30 minutes reperfusion.
n=3-5; @: P<0.05 vs Control.

Extracellular signal-regulated kinase p42/p44

Ischaemia suppressed the phosphorylation of both isoforms of ERK, while OA treatment exerted no additional effects to this (Figure 4.12: Phosphorylated protein relative to total: ERK p42: Control: 1.00±0.01 AU vs. 20 GI: 0.30±0.05 AU and PreOA: 0.32±0.07 AU; and ERK p44: Control: 1.00±0.15 AU vs. 20 GI: 0.10±0.04 AU and PreOA: 0.10±0.03 AU, n=3-5; P<0.05).

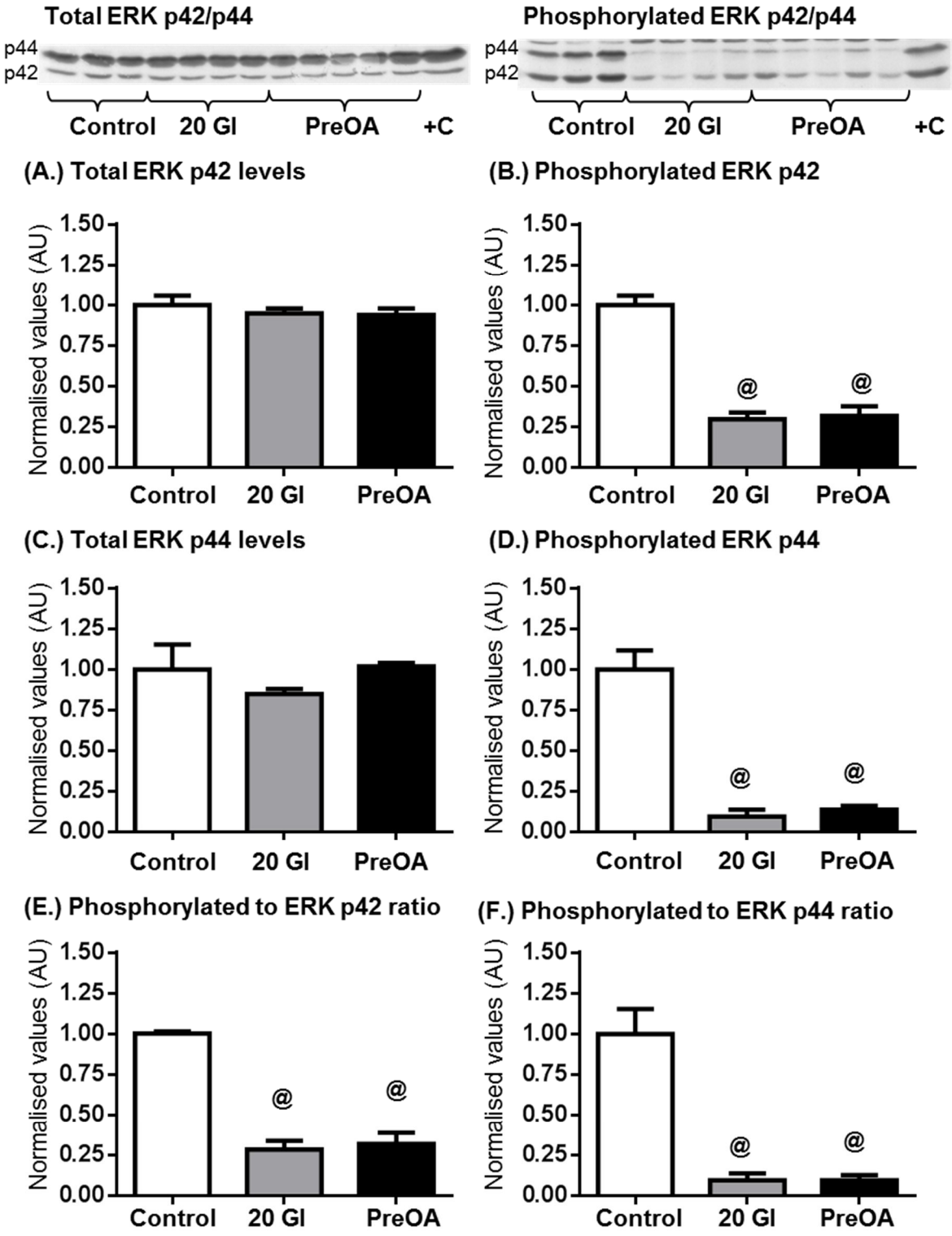


Figure 4.12. The effect of pretreatment with OA on ERK p42/p44 at 20 minutes GI. Ischaemia clearly dephosphorylated ERK p42/p44. Pretreatment with OA failed to elicit any effects on protein phosphorylation or expression.
+C: a positive control, i.e. a heart exposed to 20 minutes GI follwed by 30 minutes reperfusion. n=3-5; @: P<0.05 vs Control.

Equal loading: β-Tubulin

To assess loading, a randomly selected membrane was probed for β-Tubulin as a quality control sample for the whole series of blots. No significant differences between groups were observed (Figure 4.13).

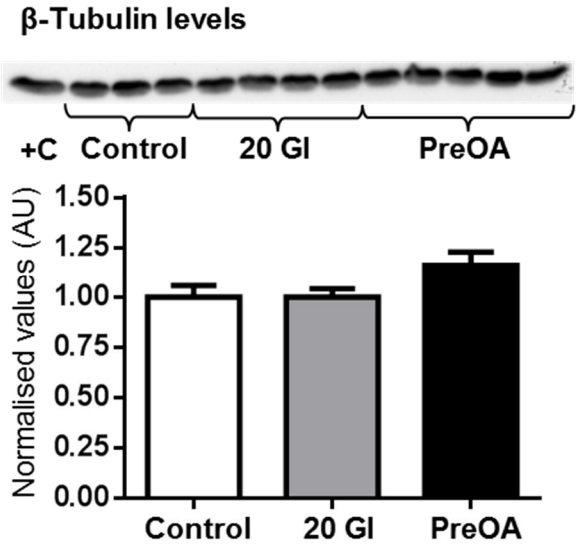


Figure 4.13. Loading control for the effects of PreOA at 20 minutes GI. β-Tubulin was used as loading control for the determination of the kinase profiles associated with pretreatment with OA. n=3-5.

Protein profiles after 20 minutes of GI and 5 minutes reperfusion

Protein phosphatase 2A

Corresponding with the changes we observed in our initial characterization of PP2A during ischaemia and reperfusion (Chapter 3), the levels of PP2A-A was reduced relative to control values at 5 minutes reperfusion (Control: 0.98±0.05 AU vs. 5 Repf: 0.75±0.05 AU, n=4; $P<0.05$) (Figure 4.14). Pre-ischaemic treatment with OA however had no effect on the levels of PP2A-C or –A.

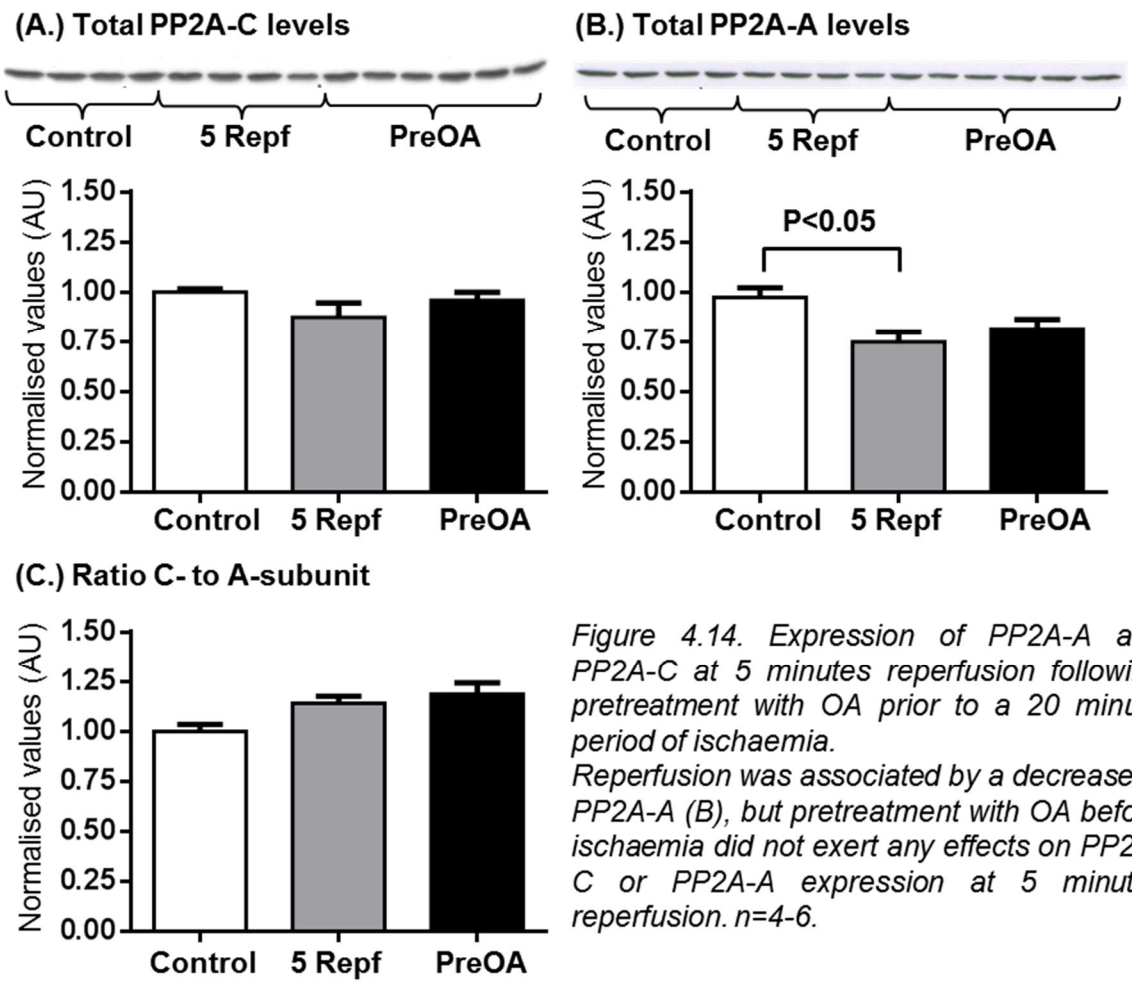


Figure 4.14. Expression of PP2A-A and PP2A-C at 5 minutes reperfusion following pretreatment with OA prior to a 20 minute period of ischaemia.
Reperfusion was associated by a decrease in PP2A-A (B), but pretreatment with OA before ischaemia did not exert any effects on PP2A-C or PP2A-A expression at 5 minutes reperfusion. n=4-6.

Okadaic acid administration however exerted an effect on the posttranslational modification of PP2A-C (Figure 4.15). On the one side OA enhanced the phosphorylation of PP2A-C (Control: 1.00±0.08 AU vs. PreOA: 2.33±0.23 AU, n=4-6; P<0.05), even to the degree that absolute phosphorylation was greater in the PreOA group than 5 minutes reperfusion alone (5 Repf: 1.35±0.18 AU vs. PreOA: 2.27±0.25 AU, n=4-6; P<0.05). Direct comparison of the phosphorylated to total ratio at 5 minutes reperfusion alone to PreOA also showed a significant difference (5 Repf: 1.55±0.21 AU vs. PreOA: 2.33±0.23 AU, n=4-6; T-test: P<0.05). On the other side OA reduced the methylation of PP2A-C relative to control (nonmethylated to total ratio: Control: 1.00±0.19 AU vs. PreOA: 2.27±0.19 AU, n=4-5; P<0.05).

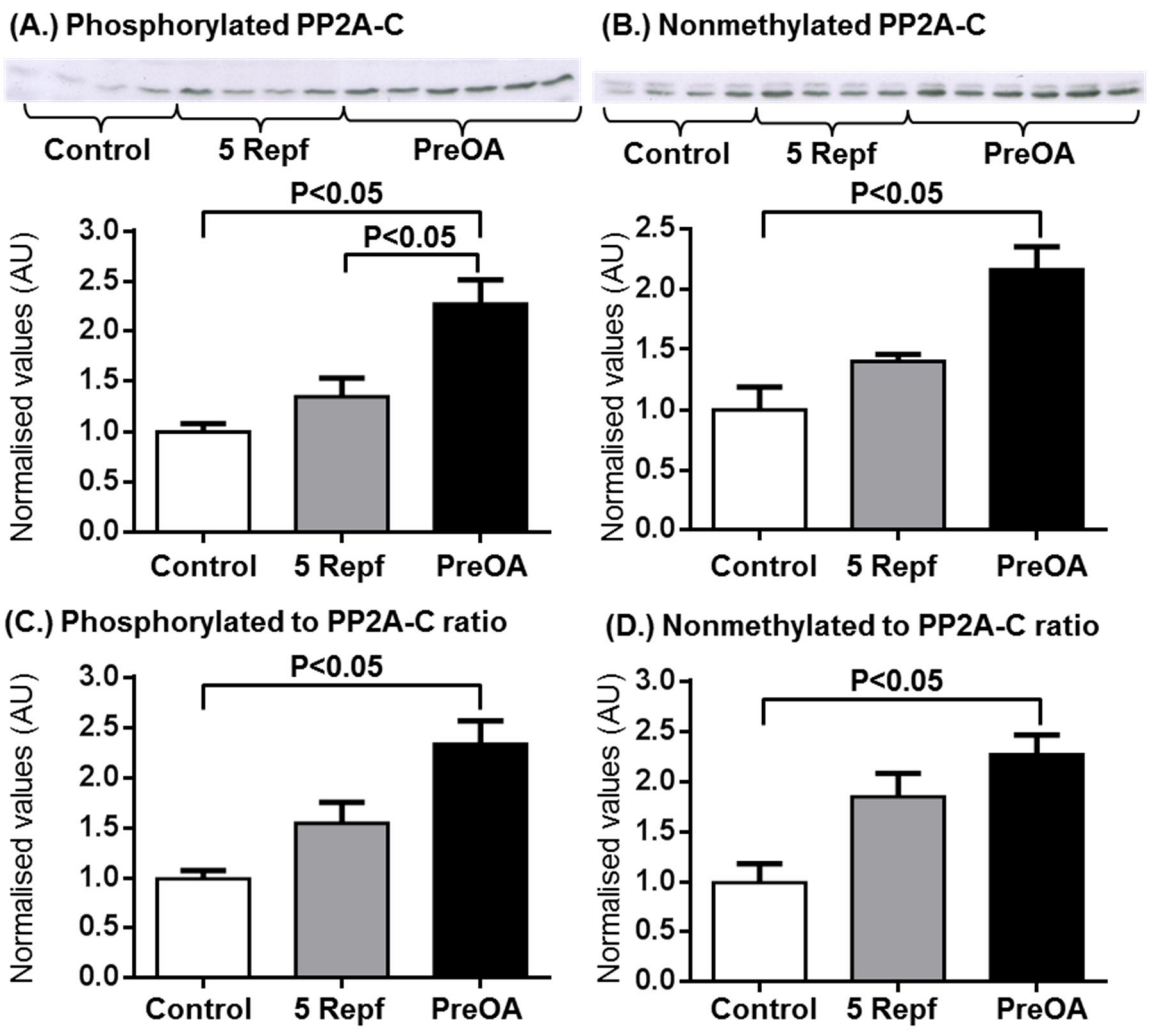


Figure 4.15. Phosphorylation and methylation of PP2A-C in hearts treated with OA prior to 20 minutes GI followed by 5 minutes reperfusion.
OA treatment increased the phosphorylation of PP2A-C (A & C), while reducing the methylation of the subunit (B & D). n=4-6.

As seen at 20 minutes GI, these data do not always correspond with the data shown and discussed in Chapter 3 (Figure 3.21), probably because of the much smaller sample size utilized for these experiments.

PP2A inhibition prior to ischaemia therefore leads to an increase in PP2A-C phosphorylation and a reduction in methylation at 5 minutes reperfusion.

Protein kinase B (Akt)

Despite a relative degree of fluctuation in the data (Figure 4.16) OA treatment was associated with an increase in the phosphorylation of PKB/Akt relative to control values (Absolute phosphorylated: Control: 1.00±0.23 AU vs. PreOA: 2.35±0.21 AU, n=4-6; P<0.05; and phosphorylated to total ratio: Control: 1.00±0.29 AU vs. PreOA: 2.25±0.27 AU, n=4-6; P<0.05 with Dunnett's correction). This data therefore shows that the inhibition of PP2A permits an increase in the phosphorylation of PKB/Akt in this setting.

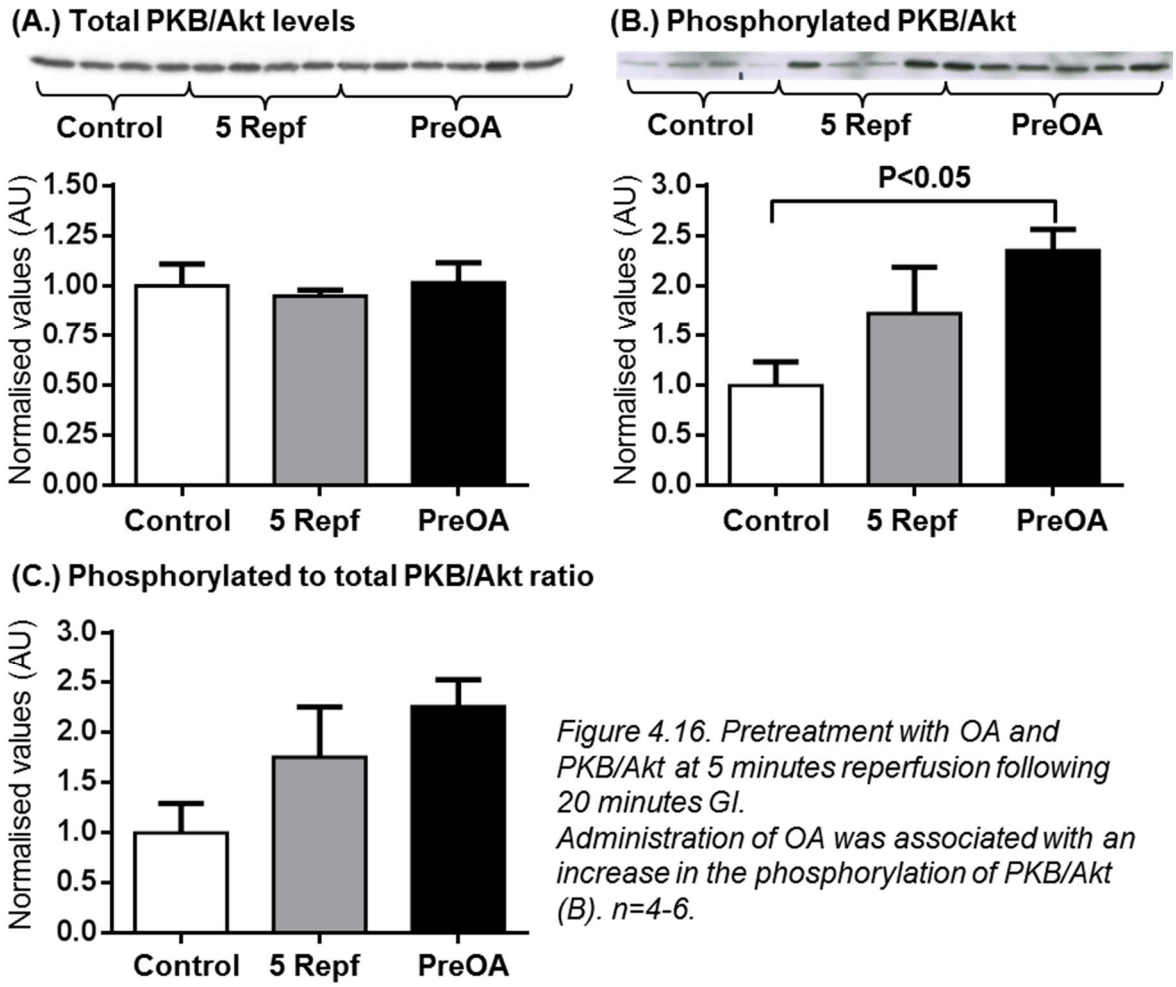


Figure 4.16. Pretreatment with OA and PKB/Akt at 5 minutes reperfusion following 20 minutes GI.
Administration of OA was associated with an increase in the phosphorylation of PKB/Akt (B). n=4-6.

Glycogen synthase kinase-3β

Corroborating the effects we observed concerning the increased phosphorylation of PKB/Akt, we found that OA administration increased the degree of phosphorylation of GSK-3β relative to the total amount of protein (Figure 4.17: Control: 1.00±07 AU vs. PreOA: 4.70±1.06 AU, n=4-6; P<0.05). As was the case at 20 minutes GI, the lack of statistical significance in the relatively blatant difference between 5 minutes reperfusion and PreOA is due to the variance within the data. Maybe it is more accurate to consider the fact that only 50% of untreated hearts which received reperfusion alone were phosphorylated, while 100% of the hearts in the PreOA group were phosphorylated relative to control (Figure 4.17).

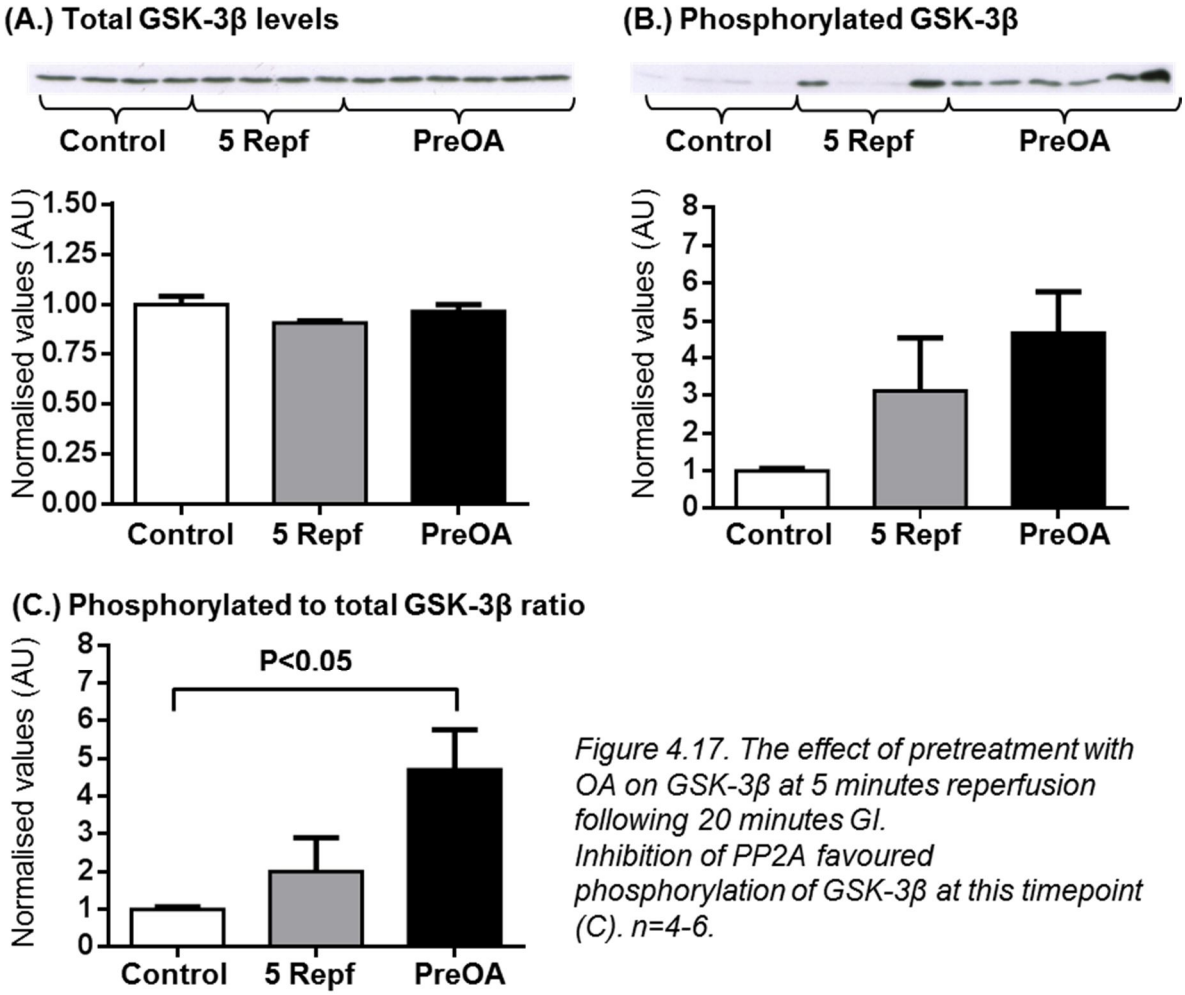


Figure 4.17. The effect of pretreatment with OA on GSK-3β at 5 minutes reperfusion following 20 minutes GI. Inhibition of PP2A favoured phosphorylation of GSK-3β at this timepoint (C). n=4-6.

p38 Mitogen activated protein kinase

Although visual inspection of the phosphorylated p38 MAPK blot would seem to indicate significant differences, at least between the control and OA treated groups, the large degree of variation in the data precludes statistical signifcance (Figure 4.18).

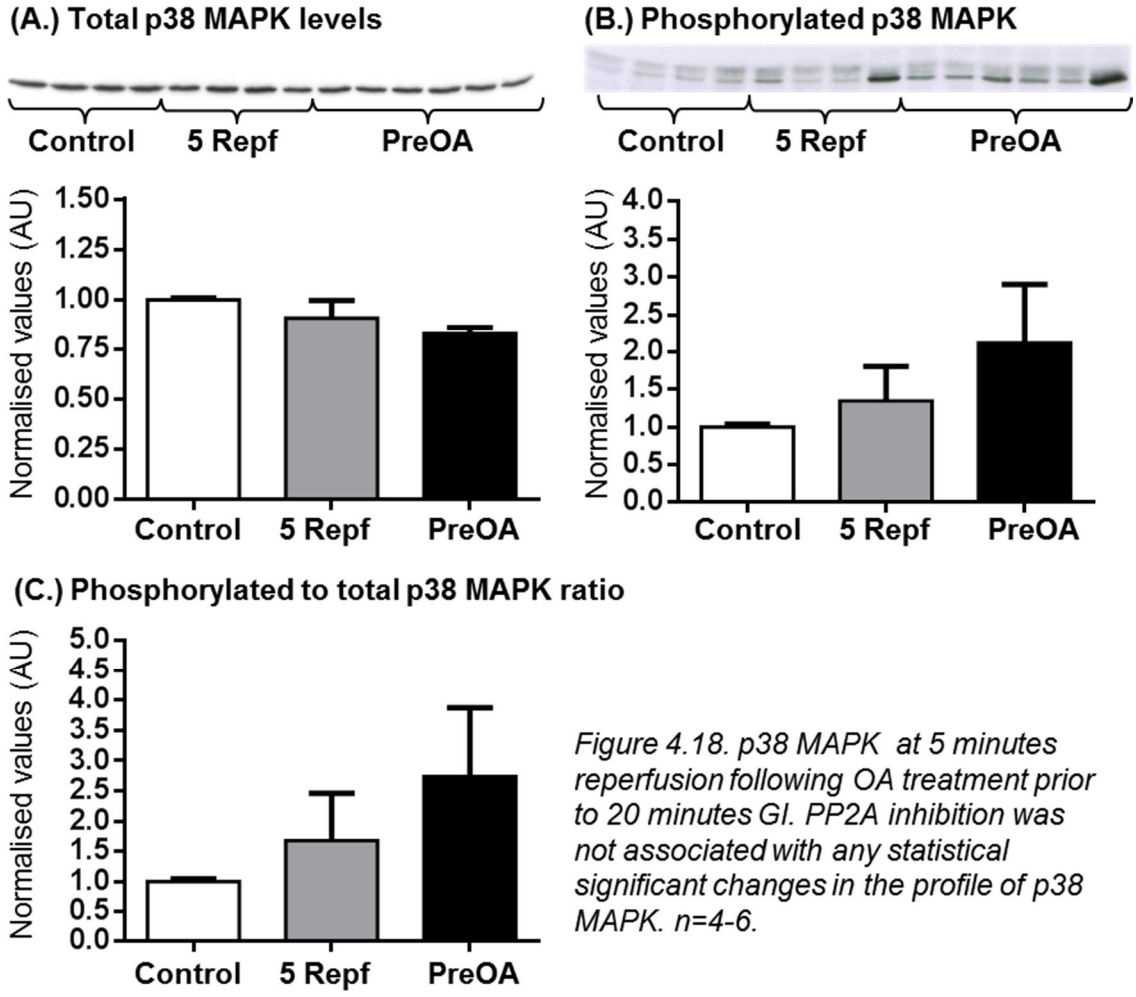


Figure 4.18. p38 MAPK at 5 minutes reperfusion following OA treatment prior to 20 minutes GI. PP2A inhibition was not associated with any statistical significant changes in the profile of p38 MAPK. n=4-6.

Extracellular signal-regulated kinase p42/p44

At 5 minutes reperfusion the group treated with OA prior to sustained ischaemia revealed a very outspoken difference from control and reperfusion alone in that it was associated with an increase in the absolute phosphorylation levels of ERK p42 (control: 1.00±0.15 AU and 5 Repf: 1.20±0.24 AU vs. PreOA: 6.18±0.55 AU, n=4-5; $P<0.05$), as well as ERK p44 (control: 1.00±0.12 AU and 5 Repf: 1.33±0.33 AU vs. PreOA: 3.56±0.64 AU, n=4-5; $P<0.05$) (Figure 4.19). Although these differences persisted for ERK p42 when expressed relative to total protein (control: 1.03±0.19 AU and 5 Repf: 1.70±0.46 AU vs. PreOA: 6.54±0.80 AU, n=4-5; $P<0.05$), it remained significant for only the comparison between control and PreOA for ERK p44 (control: 1.00±0.08 AU vs. PreOA: 3.96±0.88 AU, n=4-5; $P<0.05$). This data definitely places PP2A as an important negative regulator of ERK p42/p44, especially p42, at the onset of reperfusion.

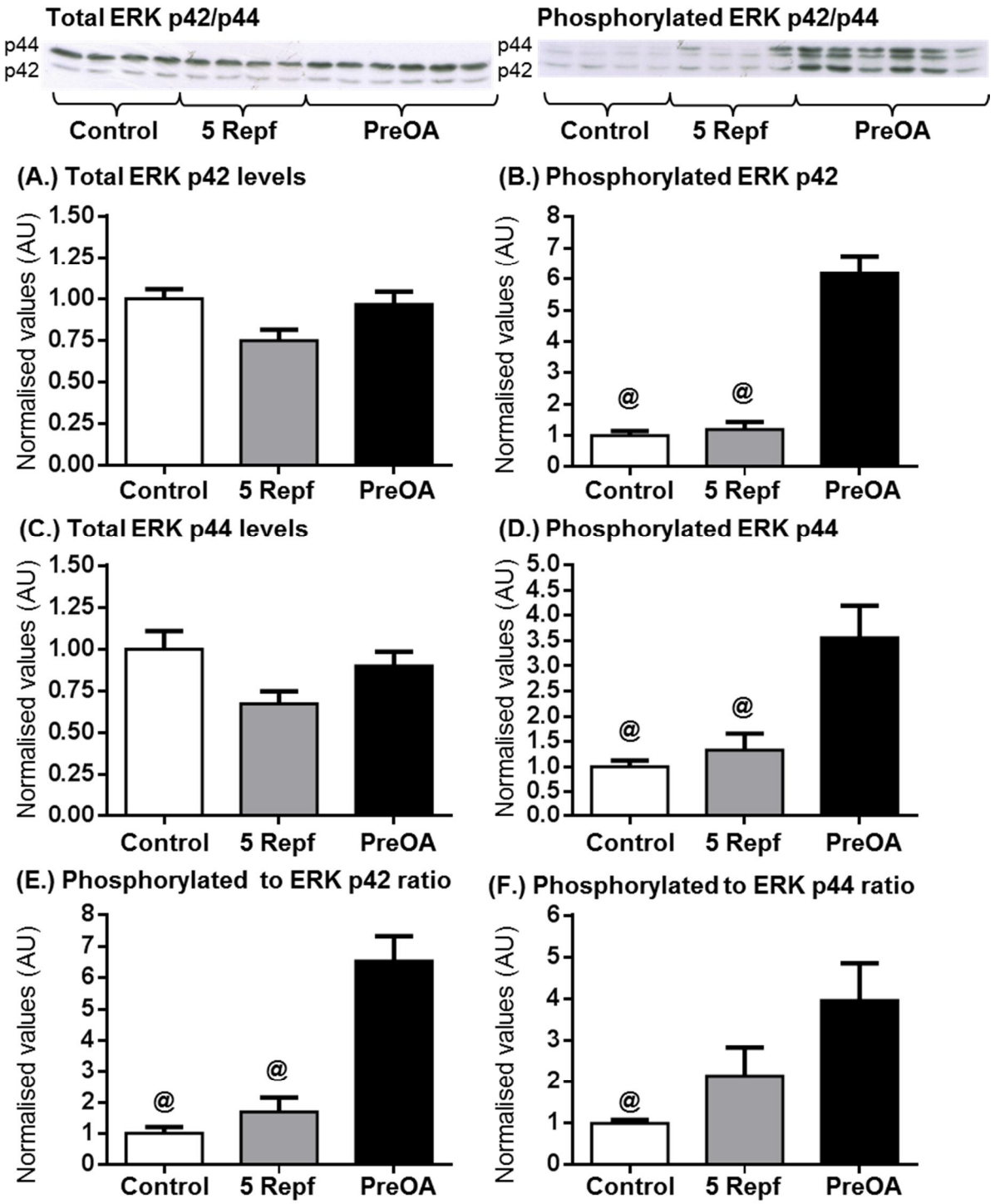


Figure 4.19. The effect of treatment with OA on ERK p42/p44 after 20 minutes GI and 5 minutes reperfusion.
PP2A inhibition prior to ischaemia elicited a robust increase in the phosphorylation of both ERK p42 and p44 at 5 minutes reperfusion. n=4-6; @: P<0.05 vs PreOA.

Equal loading: β-Tubulin

To assess loading, a randomly selected membrane was probed for β-Tubulin. No significant differences between groups were observed (Figure 4.20).

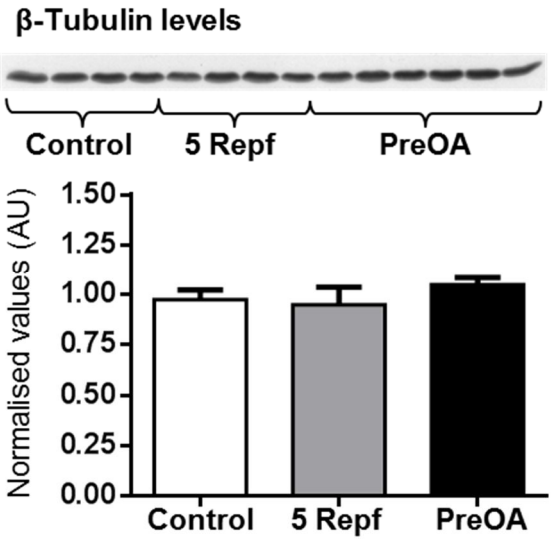


Figure 4.20. Loading control for the effects of PreOA at 5 minutes reperfusion.
β-Tubulin was used as loading control for the determination of the kinase profiles associated with pretreatment with OA. n=4-6.

Protein profiles after 20 minutes of GI and 10 minutes reperfusion

Protein phosphatase 2A

Although PP2A inhibition failed to induce any changes in the protein levels of PP2A-C or –A (Figure 4.21), we made the surprising observation that OA treatment was associated with a reduction in the ratio of PP2A-A to PP2A-C (Control: 1.00±0.07 AU vs. PreOA: 0.84±0.03 AU, n=3-6; P<0.05). In view of the fact that this shift in the ratio of PP2A-C to –A was very slight, was not associated with significant changes in total protein levels of either and was also not part of a larger pattern, it seems very doubtful if this is of biological consequence despite its statistical significance.

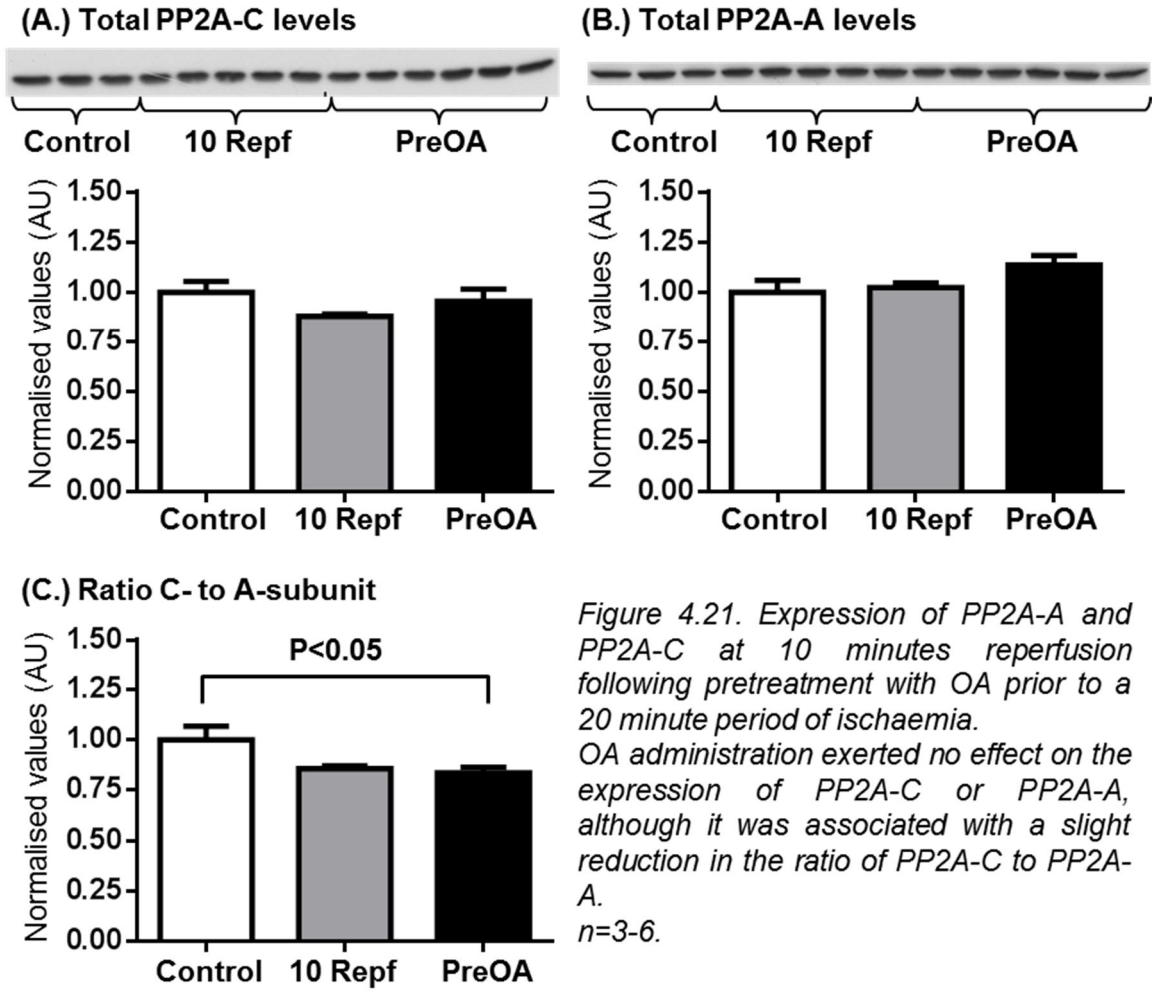


Figure 4.21. Expression of PP2A-A and PP2A-C at 10 minutes reperfusion following pretreatment with OA prior to a 20 minute period of ischaemia.
OA administration exerted no effect on the expression of PP2A-C or PP2A-A, although it was associated with a slight reduction in the ratio of PP2A-C to PP2A-A.
n=3-6.

Furthermore, OA treatment also failed to elicit any changes in the phosphorylation of PP2A-C relative to 10 minutes reperfusion alone (Figure 4.22), although both were significantly elevated in comparison to control (Phosphorylated to total ratio: Control: 1.00±0.13 AU vs. 10 Repf: 1.50±0.11 AU and PreOA: 1.54±0.05 AU, n=3-5; P<0.05). OA was however associated with a reduction in methylation in comparison to control (Control: 1.00±0.05 AU vs. PreOA: 1.42±0.07 AU, n=3-6; P<0.05).

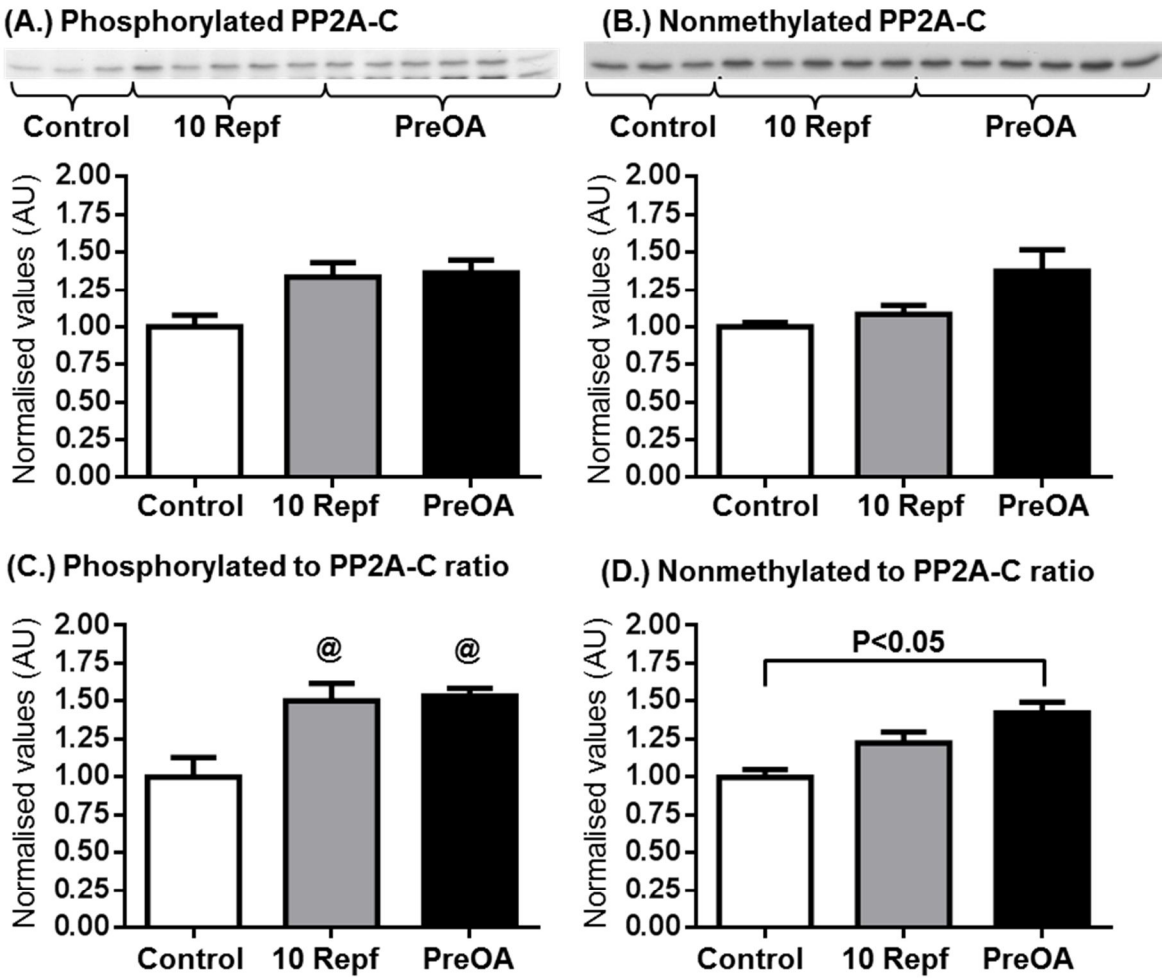


Figure 4.22. Phosphorylation and methylation of PP2A-C in hearts treated with OA prior to 20 minutes GI followed by 10 minutes reperfusion.
OA administration induced a reduction in methylation in comparison to control (D), while phosphorylation was increased in both the reperfusion alone, as well as PreOA groups (C). n=3-6; @: P<0.05 vs Control.

Besides this effect of OA treatment to favour demethylation, PP2A inhibition exerted no further effect at 10 minutes reperfusion.

Protein kinase B (Akt)

By 10 minutes reperfusion the level of phosphorylation of PKB/Akt was signifcantly elevated compared to control, with PP2A inhibition making no difference in this elevation (Figure 4.23: phosphorylated to total ratio: Control: 1.00±0.12 AU vs. 10 Repf: 4.03±0.27 AU and PreOA: 3.04±0.28 AU, n=3-6; P<0.05). It is possible that the level of phosphorylation due to reperfusion *per se* had reached an optimal level and PP2A inhibition could not enhance it any further.

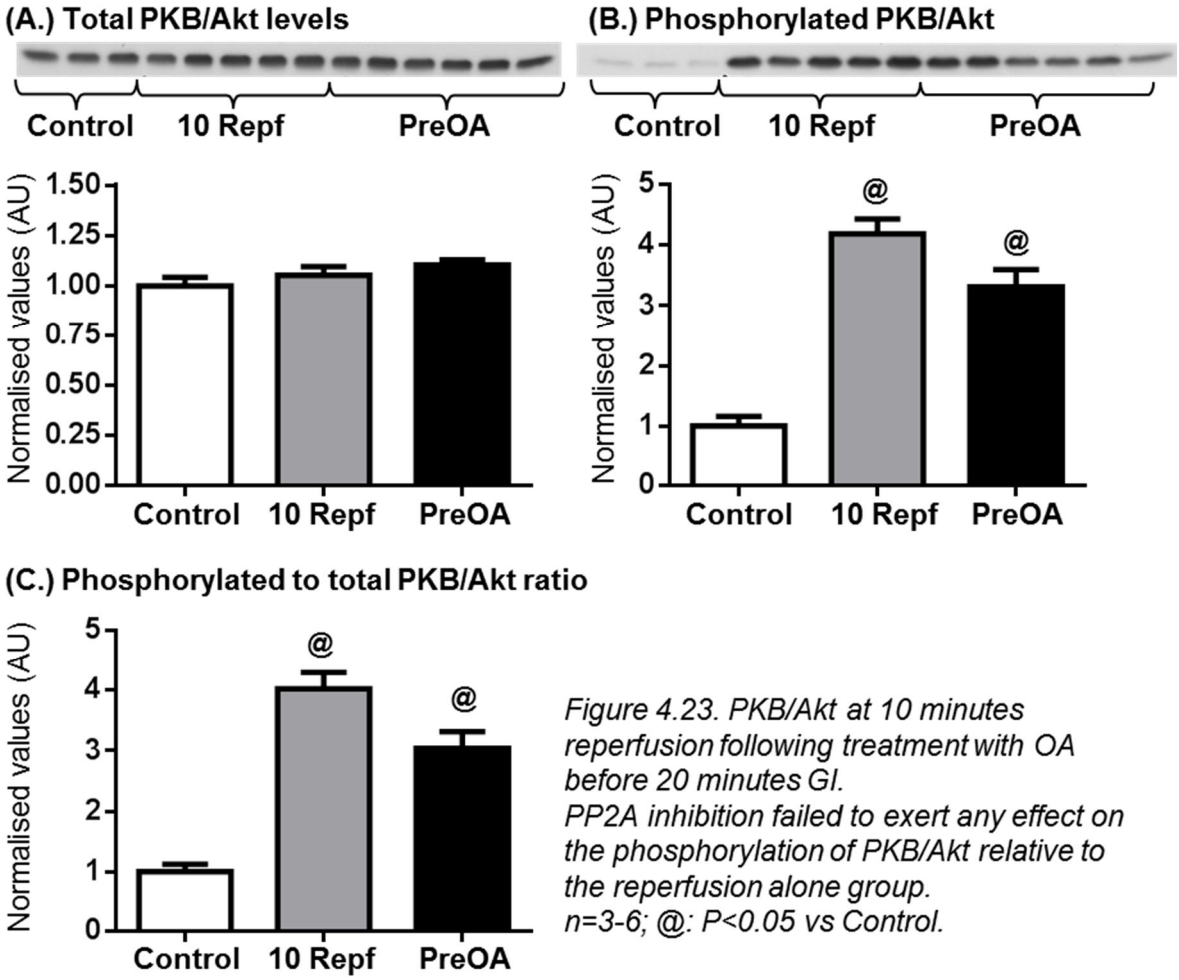


Figure 4.23. PKB/Akt at 10 minutes reperfusion following treatment with OA before 20 minutes GI.
PP2A inhibition failed to exert any effect on the phosphorylation of PKB/Akt relative to the reperfusion alone group.
n=3-6; @: P<0.05 vs Control.

Glycogen synthase kinase-3β

PP2A inhibition was not associated with any statistically signifcant changes in the phosphorylation of GSK-3β (Figure 4.24). However a seemingly obvious increase in the phosphorylation of GSK-3β in the reperfusion alone group compared to control was observed. Again it is the degree of variability in the data which precludes statistical significance. It is however noteworthy that while none of the three control hearts were phosphorylated at this time, at least three of the five hearts in the reperfusion group and two of the six in the PreOA group were phosphorylated. Despite this, it seems clear that OA treatment before ischaemia did not influence the phosphorylation of GSK-3β at 10 minutes reperfusion.

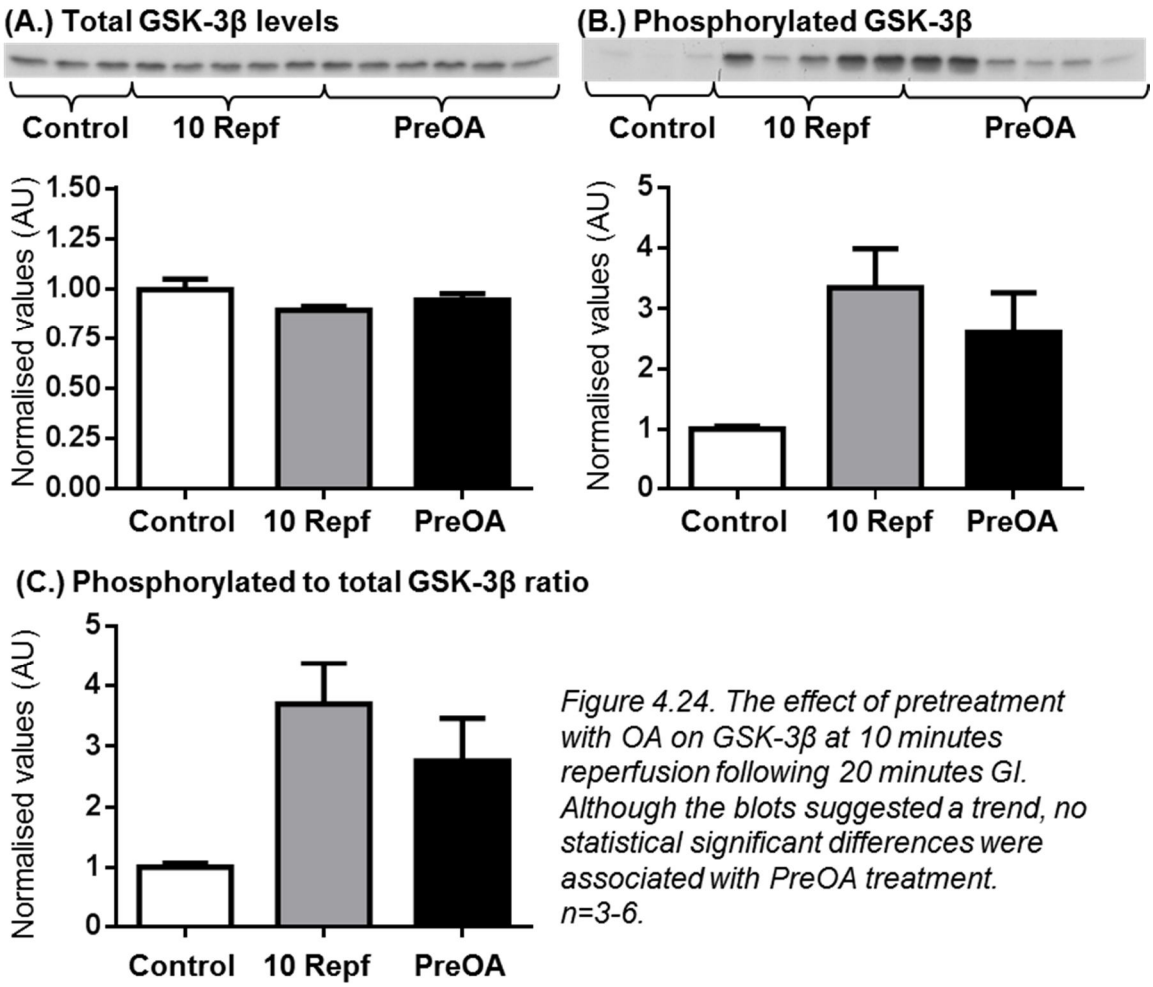


Figure 4.24. The effect of pretreatment with OA on GSK-3β at 10 minutes reperfusion following 20 minutes GI. Although the blots suggested a trend, no statistical significant differences were associated with PreOA treatment. n=3-6.

p38 Mitogen activated protein kinase

PP2A inhibition was not associated with any changes in the phosphorylation of p38 MAPK (Figure 4.25). Ten minutes reperfusion on its own was however associated with a significant reduction in total p38 MAPK levels (Control: 1.00±0.09 AU vs. 10 Repf: 0.52±0.08 AU, n=3; $P<0.05$). This has not been reported before. Investigation of the membrane itself also casts doubt upon this observation, since it might be poor antibody binding (primary or especially secondary) which might explain this very unexpected result. In this regard, there are especially two bands in the 10 minutes reperfusion group which seems faded in comparison to the other bands in the group.

As observed for GSK-3β, the lack of statistical significance regarding phosphorylation does not seem to correspond to the actual results on the membrane, where all of the hearts in the reperfusion group show phosphorylation compared to none of the hearts in the control group. Direct comparison of these two groups with a T-test reveals a significant difference (Control: 1.00±0.03 AU vs. 10 Repf: 1.66±0.18 AU, n=3-5; $P<0.05$). Irrespective of this though, it is evident that OA treatment did not impact on the phosphorylation of p38 MAPK.

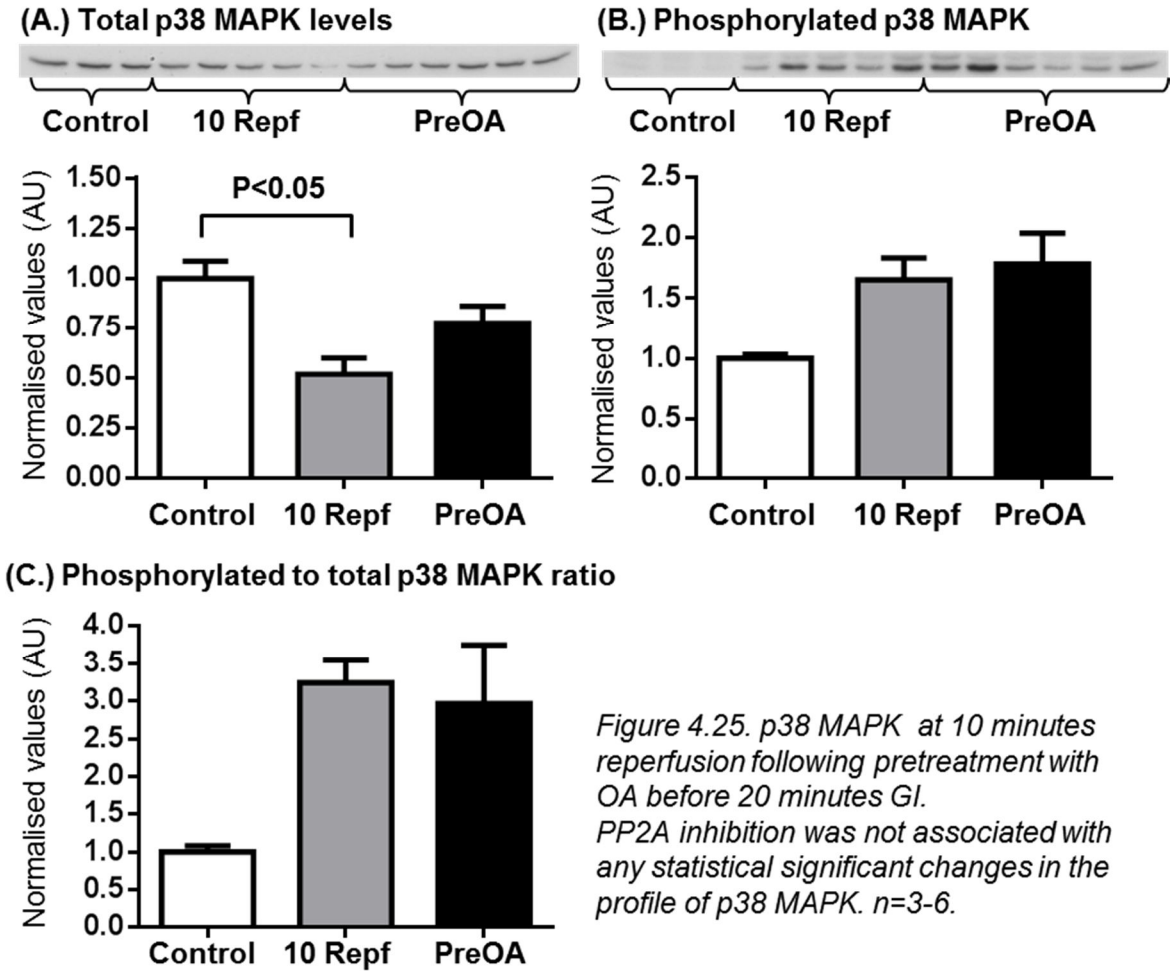


Figure 4.25. p38 MAPK at 10 minutes reperfusion following pretreatment with OA before 20 minutes GI. PP2A inhibition was not associated with any statistical significant changes in the profile of p38 MAPK. n=3-6.

Extracelllular signal-regulated kinase p42/p44

After the outspoken effect of OA treatment on ERK p42/p44 at 5 minutes reperfusion, it seems as if PP2A is of less importance at 10 minutes reperfusion (Figure 4.26). The phosphorylation of ERK p42 was significantly elevated at 10 minutes reperfusion in comparison to control, but unaffected by PP2A inhibition (ratio of phosphorylated to total protein: Control: 1.00±0.11 AU vs. 10 Repf: 3.08±0.30 AU and PreOA: 2.79±0.30 AU, n=3-6; $P<0.05$). No statistically signifcant differences were observed for ERK p44.

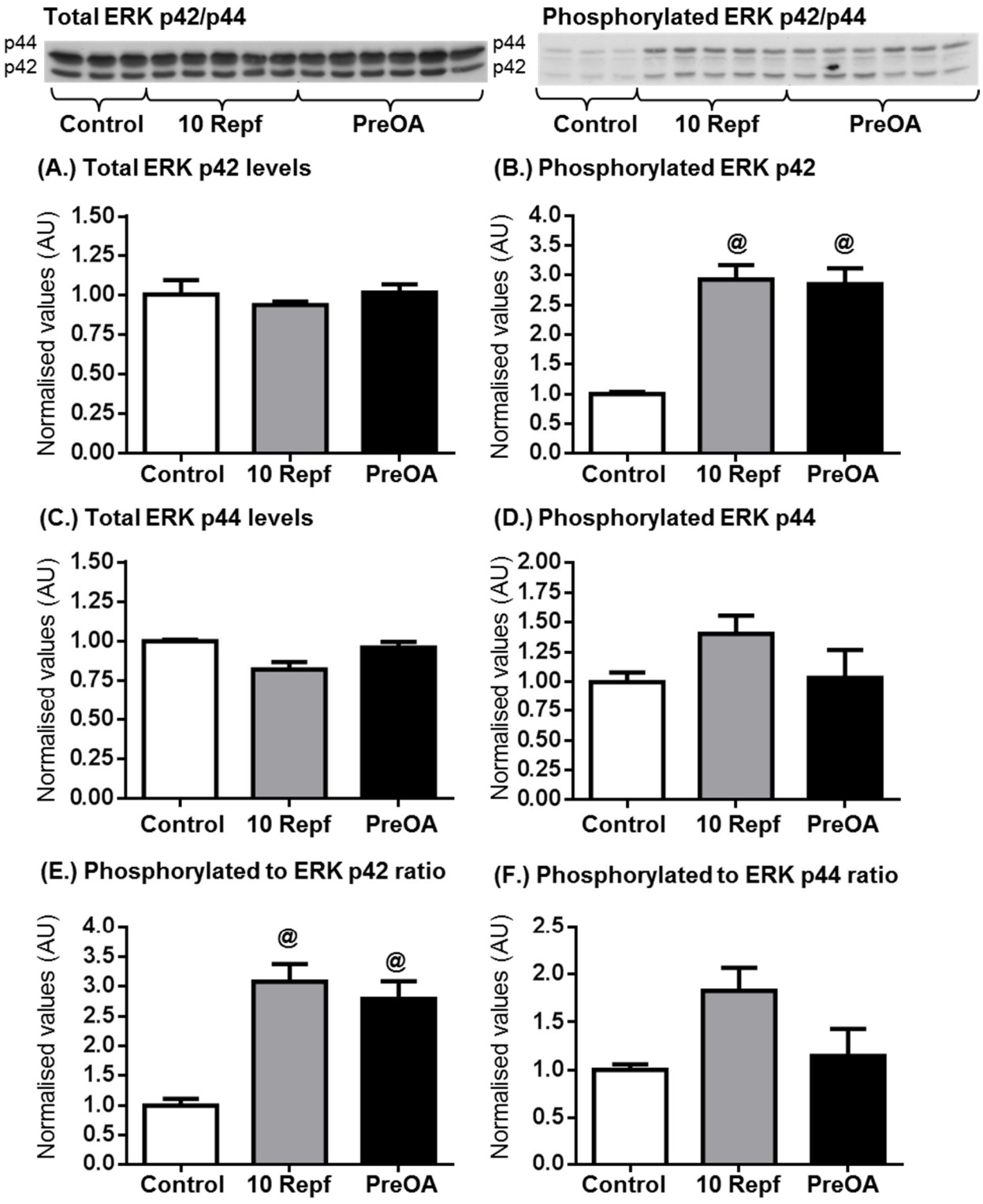


Figure 4.26. ERK p42/p44 at 10 minutes reperfusion, following OA administration and 20 minutes of GI.
Interestingly ERK p42 and p44 followed different patterns. Reperfusion alone increased the phosphorylation of p42, with PP2A inhibition failing to exert any effect (B and E), while no significant differences were seen for ERK p44.
n=3-6; @: P<0.05 vs Control.

Equal loading: β-Tubulin

To assess loading, a randomly selected membrane was probed for β-Tubulin as a representative test for the whole series of blots. No significant differences between groups were observed (Figure 4.27).

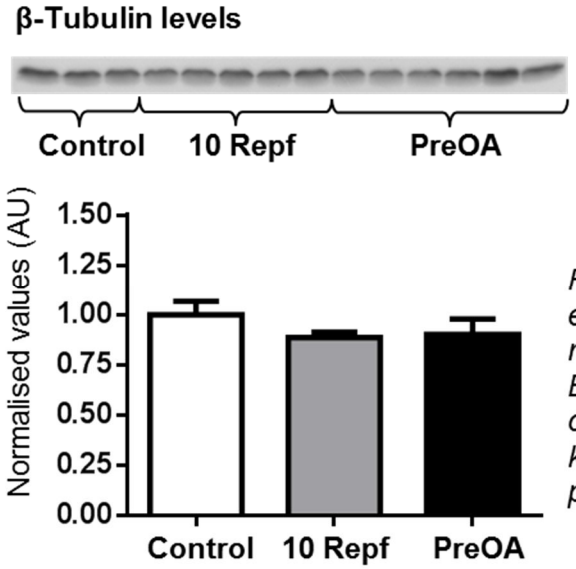


Figure 4.27. Loading control for the effects of PreOA at 10 minutes reperfusion.
B-Tubulin was used as loading control for the determination of the kinase profiles associated with pretreatment with OA. n=3-6.

Co-immunoprecipitation with PP2A-C at 5 minutes reperfusion

Western blotting of PP2A, the RISK pathway and p38 MAPK at the end of ischaemia and initial reperfusion, with and without pre-ischaemic treatment with OA, identified 5 minutes reperfusion as a period during which PP2A inhibition was associated with changes in phosphorylation of almost all proteins monitored: PP2A itself (Figure 4.15), PKB/Akt (Figure 4.16), GSK-3β (Figure 4.17) and ERK p42/p44 (Figure 4.19). Our data therefore point to 5 minutes reperfusion as a period of dynamic interaction between PP2A and its substrates. We wanted to determine if the elevation in phosphorylation of these proteins, associated with PP2A inhibition, were due to direct interactions with PP2A or due to PP2A involvement upstream of these kinases. To address this we immunoprecipitated PP2A-C from lysates prepared from the same isolated hearts which were used for the initial Western blotting experiments. We then probed the immunoprecipitate for our kinases of interest to determine if they were directly and physically associated with PP2A-C.

We included a positive control for the Western blotting component of the experiment, which was simply a normal, unprecipitated lysate, exposed to 20 minutes ischaemia and 5 mintues reperfusion. A lysate was also exposed to the precipitation protocol, but without actual exposure to the anti-PP2A-C antibody used for precipitation. This sample was used as a negative control and served to indicate background signal. For each sample analysed we therefore had to subtract this negative control signal, or alternatively the lowest signal on the blot (which per implication then had to be representative of the background signal), from the signal measured. As described in Chapter

2, this corrected value was then expressed relative to the signal of the primary precipitation target, namely PP2A-C.

Using the protocol described in Chapter 2 we succeeded in precipitating PP2A-C from the lysate, as can be judged by the strong signal for PP2A-C in the precipitate relative to even the positive control in Figure 4.28. No significant differences between groups were evident when probing for PP2A-C.

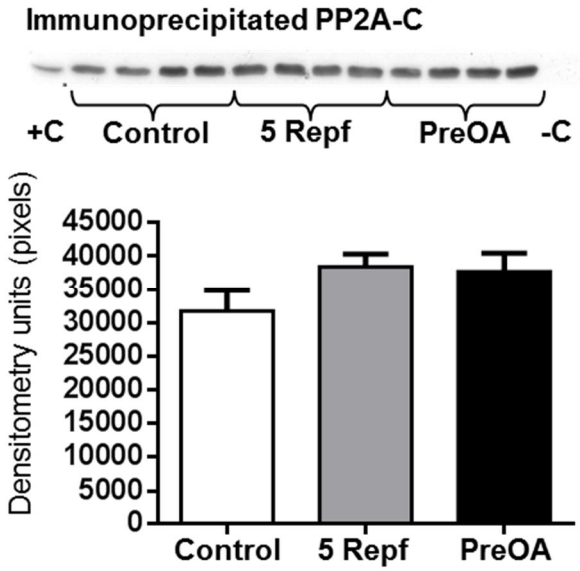


Figure 4.28. Levels of PP2A-C immunoprecipitated from heart tissue exposed to 20 minutes GI followed by 5 minutes reperfusion, in the presence and absence of pre-ischaemic OA administration. Lysate from a heart exposed to 20 minutes GI and 5 minutes reperfusion which was not exposed to the precipitation protocol was used as a positive control. The negative control was a lysis sample exposed to the precipitation protocol but in the absence of the precipitation antibody. It therefore served as an indicator of background signal. n=4.

The scaffold subunit for PP2A-C (PP2A-A)

To confirm successful co-immunoprecipitation we also probed for PP2A-A (Figure 4.29), since this subunit has been found to almost always be associated with PP2A-C in forming the core dimer. Surprisingly, we found that a direct comparison of the 5 minute reperfusion group with PreOA using an unpaired T-test showed a significant difference between these groups (5 Repf: 0.37±0.08 AU vs. PreOA: 0.02±0.01 AU, n=3-4; $P<0.01$). It therefore seems that OA interferes with the stability of the core enzyme, leading to a dissociation of PP2A-A from PP2A-C.

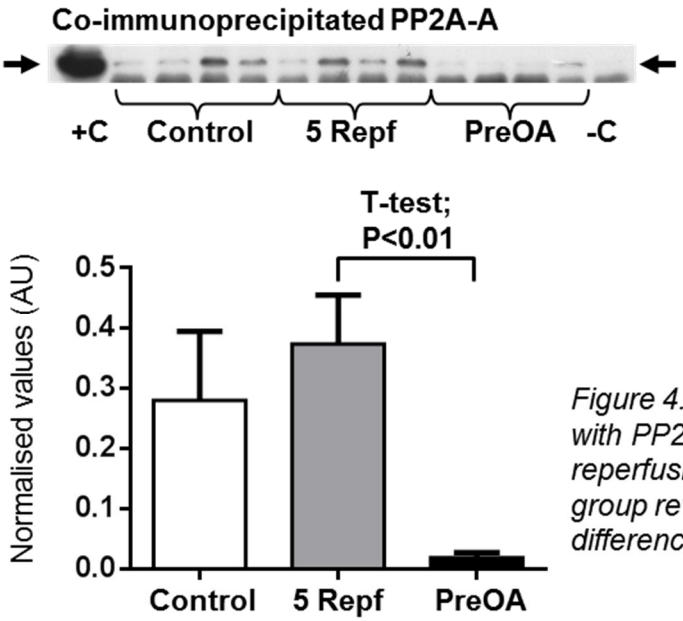


Figure 4.29. PP2A-A co-immunoprecipitated with PP2A-C. Comparison of the 5 minute reperfusion group with the OA treatment group revealed a statistically significant difference. n= 3-4.

Protein kinase B (Akt)

Co-precipitation with PKB/Akt is shown in Figure 4.30. Although we found PKB/Akt associated with PP2A-C, this interaction was not statistically influenced by PP2A inhibition – although a strong trend showing reduced interaction was evident.

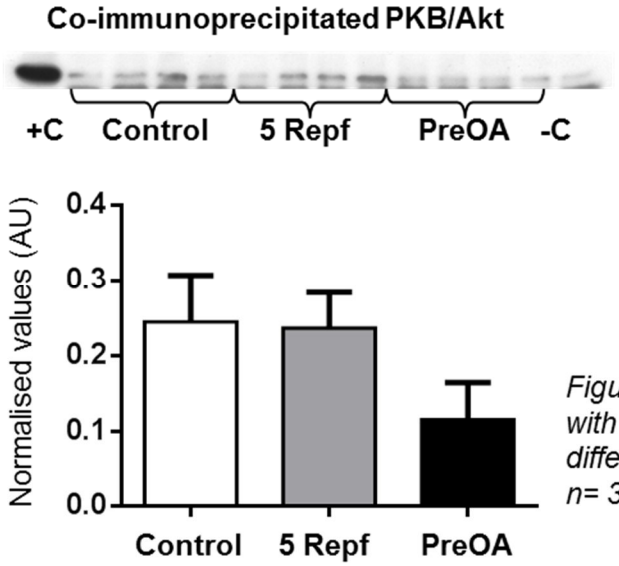


Figure 4.30. PKB/Akt co-immunoprecipitated with PP2A-C. There were no significant differences between any of the groups. n= 3-4.

Glycogen synthase kinase-3β

As was the case for PKB/Akt, although GSK-3β could be precipitated together with PP2A-C, OA administration failed to exert any effect on this interaction (Figure 4.31).

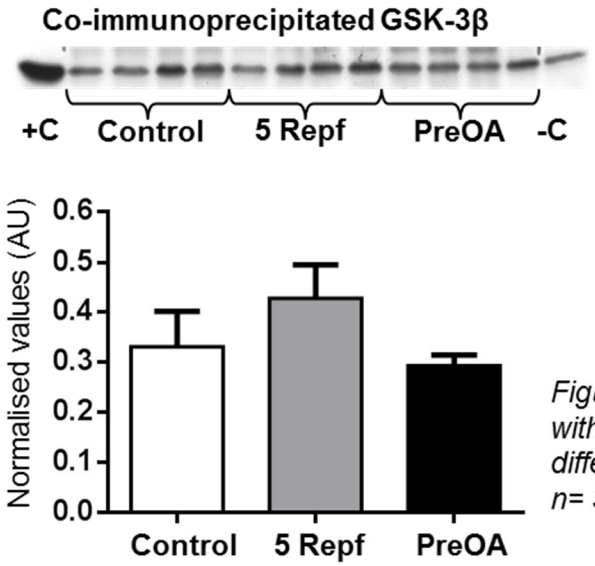


Figure 4.31. GSK-3β co-immunoprecipitated with PP2A-C. There were no significant differences between any of the groups. n= 3-4.

p38 Mitogen activated protein kinase

Surprisingly we found that not only was p38 MAPK associated with PP2A-C, but OA administration reduced this interaction compared to 5 minutes reperfusion alone (Figure 4.32: 5 minutes reperfusion: 0.27±0.03 AU vs. PreOA: 0.14±0.01 AU, n=3-4; P<0.01). This interaction between PP2A-C and p38 MAPK and the negative effect of OA administration on it was however not evident in the Western blotting data. This could possibly be explained by the fact that p38 MAPK could potentially be both upstream (as an activator) or downstream (as a substrate) of PP2A.

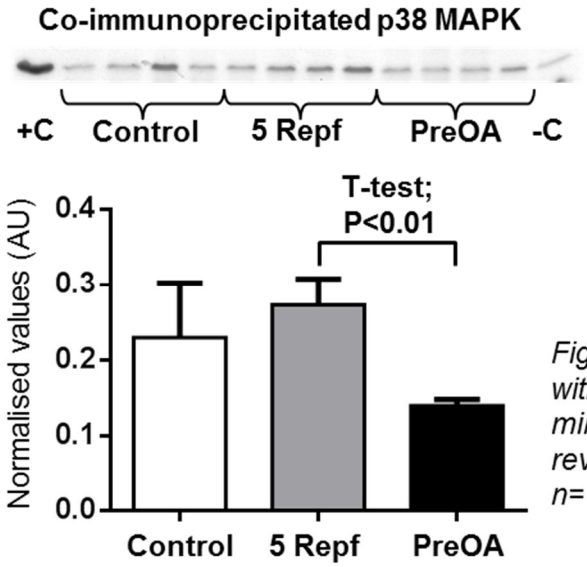


Figure 4.32. p38 MAPK co-immunoprecipitated with PP2A-C. Direct comparison of the 5 minutes reperfusion and PreOA groups revealed a statistical significance. n= 3-4.

If it is not exclusively a substrate of PP2A (as indicated by our Western blotting data), than its interaction with PP2A-C, which is implicated by our precipitation data, could be indicative of an upstream role for p38 MAPK in this setting.

Extracellular signal-regulated kinase p42/p44

Data concerning the interaction between ERK p42/p44 and PP2A-C are shown in Figure 4.33. As expected, we could precipitate ERK p42/p44 alongside PP2A-C, confirming physical interaction between these two proteins. Direct comparison of Control with PreOA revealed a reduction in the association of ERK p42 with PP2A-C in the presence of OA (Control: 0.09±0.02 AU vs. PreOA: 0.03±0.01 AU, n=4; unpaired T-test: P<0.05). Analysis of variance of the ERK p44 data also revealed a significant reduction in its association with PP2A-C in the presence of OA (Control: 0.26±0.02 AU, and 5 minutes reperfusion: 0.22±0.04 AU vs. PreOA: 0.11±0.02 AU, n=3-4; P<0.05). Taken together, this implicates a physical interaction between ERK p42/p44 and PP2A-C at 5 minutes reperfusion.

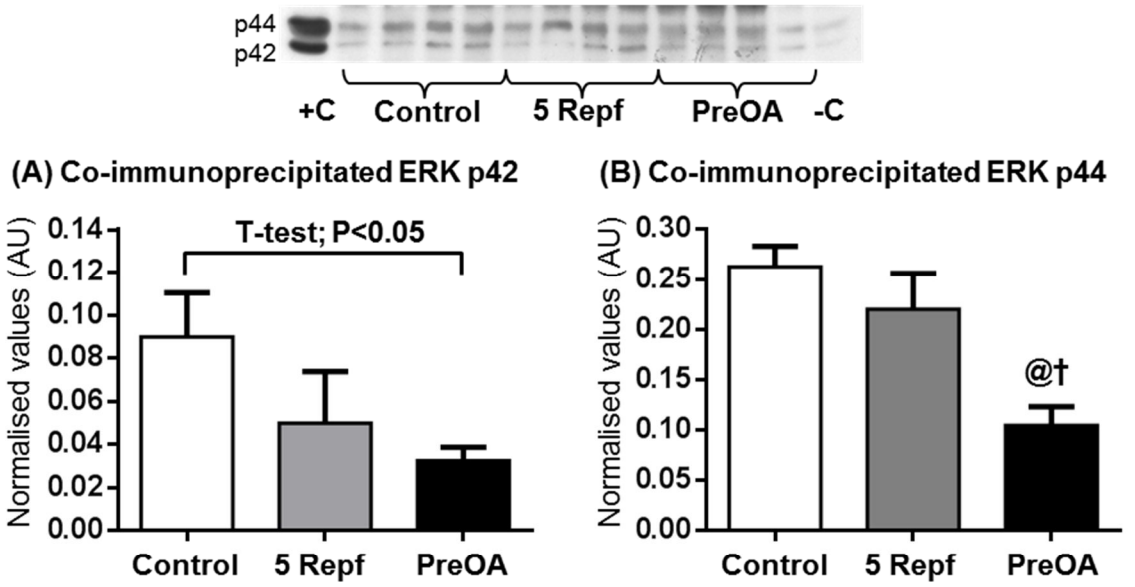


Figure 4.33. Co-immunoprecipitation of ERK p42/p44 with PP2A-C at 5 minutes reperfusion in the presence and absence of OA. Okadaic acid interfered with the association between ERK p42 and PP2A-C in comparison to control (A), while ERK p44 was signicantly less associated with PP2A-C in the PreOA group compared to both control and 5 minutes reperfusion (B).
n= 3-4; @ P<0.05 vs. Control, † P<0.05 vs. 5 minutes reperfusion.

Discussion

Having established in Chapter 3 that PP2A is recruited into the cellular response of the myocardium to I/R, the obvious next question pertains to the exact role of PP2A in the development of I/R injury. To answer this question we followed a pharmacological-based approach whereby we modulated the activity of PP2A within this setting. The consequences of PP2A activation will be addressed in Chapter 5, while in this part of the study we investigated the effects of PP2A inhibition.

Effects of PP2A inhibition on infarct size

We used 10 nM of the marine toxin okadaic acid (OA) to inhibit PP2A. As described in "Materials and methods", this concentration was chosen since it is a suitably low concentration to ensure that only PP2A is inhibited, and not PP1 as well (Bialojan & Takai, 1988; Cohen *et al.*, 1990; Dawson & Holmes, 1999; Herzig & Neumann, 2000). Others have also used similar concentrations (Ladilov *et al.,* 2002; Fan *et al.*, 2010). As a starting point for this study we administered OA directly before sustained ischaemia and/or during the onset of reperfusion to assess the effects of PP2A inhibition on IFS. We found that of the three protocols tested, only pretreatment with OA elicited a significant effect: namely a reduction in IFS (Figure 4.5). The association between protein phosphatase inhibition and cardioprotection has been made by several researchers, although most of these studies failed to distinguish between the inhibition of PP2A and PP1. Studies which utilized OA are described in table 4.4.

Our results confirm the majority of these studies, namely that pre-ischaemic treatment with OA confers cardioprotection. The use of other inhibitors such as fostriecin and calyculin A generated similar results where pretreatment and even administration during ischaemia conferred protection (Armstrong *et al.*, 1997; Weinbrenner *et al.*, 1998). Concerning specifically IFS as end-point, there are two noteworthy exceptions: Fenton and colleagues (2005) investigated the interplay between IPC mediated cardioprotection and phosphatase inhibition in aged versus young rat hearts. In their study it was only reperfusion treatment with 100 nM OA which reduced IFS in young hearts, and not pre-ischaemic treatment, while in aged hearts OA had to be administered both before and after ischaemia to elicit a similar effect. Recently, Fan and co-workers (2010) reported that treatment with 7.5 nM OA had no effect in non-preconditioned hearts on IFS. As is too often the case, there are several fundamental differences between these studies and ours which might explain the lack of consistency in results.

There are especially four differences which are important: (1.) The type of ischaemia applied: Although Fan *et al.* and ourselves utilized a similar 35 minute regional ischaemic intervention, Fenton *et al.* used 30 minutes of GI. (2.) The dose of OA administered: We used 10 nM while Fan *et al*. used 7.5 nM. It is a small difference, but might explain the lack of effect in their model, since even 10 nM is quite a low dose compared to the concentrations used in other studies. Fenton, for example, used 100 nM OA. (3.) The duration of OA administration: Fenton *et al*. administered OA over a period of 25 minutes, while Fan *et al.* and ourselves only administered OA for a duration of 10 minutes. Taking the latter two points into consideration, hearts in Fenton *et al.*'s study were exposed to much more OA prior to ischaemia, than those investigated in our study, as well as the study by Fan and co-workers. (4.) Perfusion mode: Fan *et al.* and ourselves utilized a combination of normal retrograde and work mode perfusion, while Fenton *et al.* only retrogradely perfused their hearts.

Table 4.4. Studies which have reported the use of OA in the setting of I/R.
LVdevP: Left ventricular developed pressure; HR: Heart rate; RPP: Rate pressure product.

Reference	Experimental model	End-point	OA (nM)	Administration of OA	Conclusion
Xiuhua *et al.*, 1997	Vascular smooth muscle cells exposed to 2 hours hypoxia.	Trypan blue detection of cell viability, LDH release and ATP content.	2000	10 minute pre-hypxoc treatment.	Pretreatment increased viability and ATP content in conjunction with a reduction in LDH release.
Armstrong & Ganote, 1992	Isolated rat ventricular cardiomyocytes.	Trypan blue exclusion and osmotic fragility, as well as rate of contracture.	5000 – 20 000	10 minutes prior to metabolic inhibition / SI and during intervention as well.	Metabolic inhibition: increased viability and reduced osmotic fragility. SI: increased rate of contracture, no protection visible unless corrected for the associated increase in ATP depletion in these preparations.
Barancik *et al.* 1999	*In vivo* swine heart exposed to 60 minutes RI.	IFS	600	60 minutes prior to ischaemia.	OA treatment reduced IFS.
Isotani *et al.* 2002	*In vivo* rat model of 45 minutes renal ischaemia.	Renal function and histology.	200	During early reperfusion.	Beneficial effects with regards to both function and morphology.
Ladilov *et al.*, 2002	Isolated rat hearts exposed to 60 minutes GI.	Function (LVdevP, HR, RPP and aortic pressure)	5	20 minutes pre-ischaemia	No effect on its own, but enhanced functional recovery induced by hypoxic preconditioning
	Isolated ventricular rat cardiomyocytes exposed to 60 minutes anoxia.	Ca^{2+}, Na^{+} homeostasis and cell length.	5	20 minutes pre-anoxia and during 60 minutes anoxia	No effect on its own, but enhanced hypoxic preconditioning mediated reduction in Na^{+} and Ca^{2+} overload.
Fenton *et al.*, 2005	Isolated rat hearts exposed to 30 minutes GI.	IFS	100	25 minutes prior to ischaemia, and / or first 10 minutes of reperfusion.	Pre+Post reduced IFS in aged rat hearts. Only reperfusion alone protected in young hearts (did not test Pre+Post in young hearts).
Fan *et al.*, 2010	Isolated rat hearts exposed to either 35 minutes RI or 15 or 20 minutes GI.	IFS and functional recovery	7.5	10 minutes before ischaemia or first 10 minutes of reperfusion.	In combination with IPC reperfusion treatment increased functional recovery. No effect on IFS. Pre-ischaemic treatment reduced functional recovery.
Sariahmetoglu *et al.*, 2012	Isolated rat hearts exposed to 20 minutes GI.	Mechanical function (RPP)	100 Or 10	10 minutes before ischaemia and 10 minutes into reperfusion.	100 nM OA protected against functional deterioration, while 10 nM conferred no protection.

In view of these differences it is difficult to compare our results with those generated by Fenton and colleagues, as well as Fan *et al*. This present study is much closer to the study reported by Fan *et al.* with the only major difference being the concentration of OA used, which might explain the lack of an infarct sparing effect in their experiments. In this regard it is noteworthy that combination of a reperfusion treatment of OA with an IPC intervention increased functional recovery after 20 minutes GI in Fan *et al.*'s model. This protective effect of OA in combination with IPC could imply that their dose of OA was indeed too low.

There seems to be consensus that protein phosphatase, specifically PP2A, inhibition prior to sustained ischaemia is cardioprotective. This however raises the question why reperfusion treatment alone, or combination of pre-and reperfusion treatment could not elicit similar effects. We can only speculate on the reasons for this, but it seems the following two parameters must be taken into consideration (see also table 4.4):

1.) The concentration of OA administered. This has already been referred to above. As alluded to in the "Materials and Methods" section, the cell membrane permeability and intracellular protein phosphatase content also contribute to determining an optimal dose for OA. Thus, even though the IC_{50} of OA for PP2A is in the range of 0.2-1 nM, it has been reported that doses even as high as 1 µM is necessary to effectively inhibit PP2A, while still not exerting an effect on PP1 (Favre *et al.*, 1997 – this work was done on a cancerous cell line, and given the fact that PP2A is modulated in some forms of cancer (Schöntal, 2001), these observations must be interpreted with caution). Recently Sariahmetoglu *et al.* (2012) investigated the effects of OA administration in the isolated rat heart exposed to 20 minutes GI. They measured PP2A activity in hearts exposed to either 10, or 100 nM OA and found that only 100 nM statistically reduced the activity of PP2A and it was also the dose which exerted a cardioprotective effect. On the other hand, the standard error on the data generated by 10 nM OA precluded statistical significance, and this dose also failed to elicit cardioprotection.
2.) The duration of OA administration: In their characterization of OA, Favre and colleagues (1997) reported that OA administered in a range of 10 to 100 nM inhibited PP2A in MCF7 cells over a period of 12 (100% inhibition at a dose of 100 nM) to more than 48 hours (~ 80% inhibition with 10 nM at 48 hours incubation). Jayaraj *et al.* (2009) found that 100 nM of OA inhibited PP2A by only 50% in HeLa cells following 8 hours of incubation. In line with these reports which emphasise the importance of the duration of OA exposure, it is noteworthy that some researchers working on the isolated rat heart model administered their PP2A inhibitors for relatively long periods of time. Ladilov *et al.* (2002) administered OA over a period of 20 minutes, while Weinbrenner and colleagues (1998) administered the PP2A inhibitor fostriecin for a total period of 45 minutes.

Applying these two variables to our study it seems clear that the dose we used, while probably not inhibiting PP1 and only affecting PP2A, is possibly at the lower limits of efficacy. In fact, it is possible that the significant reduction in IFS in the PreOA group could be attributed to the duration of administration: if administered 10 minutes prior to ischaemia, and assuming that the drug remains in the infarcted zone for the following 35 minutes of RI, the ischaemic portion of the heart would be exposed to OA for a total of 45 minutes. Obviously the timing of OA administration is also important, and our results, together with the majority of work published, imply that ischaemia itself, and possibly the very onset of reperfusion are important time points for the inhibition of PP2A to exert cardioprotection. During these time periods there must therefore be intracellular proteins which are normally dephosphorylated by PP2A, and which elicit pro-survival effects when phosphorylated.

This then may explain why, in this study, the relatively low dose of OA administered prior to ischaemia protected the heart. If long enough exposure to OA is the key, why did the combined pre- and reperfusion treatment with OA fail to protect the hearts? A possible answer to this lies in the balance between the degree of PP2A inhibition and the specific subpopulations of PP2A which are inhibited. It could be argued that in our model, a fine balance exists between optimal and detrimental inhibition of PP2A in terms of the degree of loss of PP2A activity, as well as the substrates affected by this. Administration of OA both before and after sustained ischaemia might disrupt this balance and negate the positive effects of PP2A inhibition associated with the pre-ischaemic administration of a PP2A inhibitor. More specifically, it could be that the loss of too much PP2A activity at the onset of reperfusion may be detrimental. This would then also explain why we did not see an infarct sparing effect when OA was administered during reperfusion alone. If this is the case, what could the potential mechanism be? The answer to this question lies outside the scope of this study and can only be speculated about. In 1993 Neumann *et al.* reported that OA can induce a positive inotropic effect in papillary muscles isolated from the guinea pig heart. They found that this effect was associated with an increase in Ca^{2+} flux through the L-type Ca^{2+} channel. Since then, PP2A has been implicated in the regulation of several Ca^{2+} regulator molecules such as PLB and the RyRs (see appropriate section in Chapter 1). It is therefore possible that the same mechanism involved in inducing a positive inotropic effect under baseline conditions could favour intracellular Ca^{2+} overload during reperfusion, thereby contributing to I/R injury.

But why then did Fenton and colleagues report an infarct sparing effect associated with the reperfusion administration of 100 nM OA in an isolated rat heart model exposed to 30 minutes GI? There are two possible explanations: First, there are fundamental differences in the experimental model utilized by Fenton *et al.* vs. ourselves: Fenton and co-workers exposed their hearts to retrograde perfusion with pacing alone, while we utilized a work heart model which presents with a different experimental platform concerning metabolic and Ca^{2+} homeostasis. Second, the

differences in dosage OA applied: It could be argued that we simply administered OA at a too low dose during reperfusion to see the same effects which Fenton and co-workers reported.

Despite the contradictory and challenging results in the literature, our data stands: OA treatment at a relatively low concentration of 10 nM prior to sustained ischaemia reduced IFS, thereby indicating that PP2A activity during ischaemia, and possibly the very onset of reperfusion is detrimental to the heart.

Effects of PP2A inhibition on functional recovery

In a model of 35 minutes regional ischaemia

Although we found that pre-ischaemic treatment with OA reduced infarct size, there was no associated increase in the functional recovery of these same hearts despite the reduction in infarct size (table 4.2). This is an unexpected result, as one would expect that the reduction in IFS would also translate in an increase in functional recovery. Although many authors have found the expected association between IFS and post-ischaemic functional capacity (Jenkins *et al.*, 1995; Salie *et al.*, 2011), an uncoupling of functional ability and IFS has also been reported by several others as well (Cohen *et al.*, 1999; Ford *et al.*, 2001; Uematsu *et al.*, 2001; Lochner *et al.*, 2003; Van Vuuren *et al.*, 2008; Huisamen *et al.*, 2012).

The most plausible explanation for the dissociation between IFS and functional recovery lies in what is fundamentally measured by these endpoints. The determination of IFS is dependent on the leakage of enzyme (dehydrogenases) from damaged cells (see Chapter 2). Thus, IFS is a measure of necrosis and possibly late apoptosis, since major damage to the cell membrane is a prerequisite for the detection of an infarcted zone. Functional recovery on the other hand is a composite endpoint which is affected by several factors including, cell death, stunning and arrhythmias. This means that even if the degree of cell death is reduced by an intervention, it will not necessarily translate into an increase in functional recovery. An excellent example of this is the phenomenon known as stunning, where an ischaemic period which is too short to induce meaningful necrosis still suppresses function (Braunwald & Kloner, 1982; Bolli, 1990). Stunning has been attributed to, amongst others, dysfunctional Ca^{2+} handling (Gross *et al.*, 1999) and might in fact be one of the most important reasons why infarct size does not necessarily match post-ischaemic function. In 2000 Cohen and co-workers reported that only when reperfusion time was extended to 3 weeks, the infarct sparing effects of an IPC protocol better matched functional recovery. They suggested that this could be due to the effects of stunning diminishing over time, as well as adaptive remodelling of the heart.

We therefore conclude that pre-ischaemic administration of 10 nM OA exerts a greater effect on PP2A-mediated dephosphoryation of proteins involved in cell survival than on other determinants of function such as stunning and arrhythmias.

In a model of 20 minutes global ischaemia

Having established that OA treatment failed to increase functional recovery in a model of RI, we wanted to determine what the effect of OA administration would be in a model specifically aimed at measuring functional recovery as primary endpoint. For these experiments we employed a model of global ischaemia. A major benefit of this model is the fact that it generates a relatively homogeneously damaged heart, thereby offering much more tissue for Western blotting analysis than RI.

Treating hearts with 10 nM OA directly prior to 20 minutes GI however failed to elicit a significant increase in functional recovery (Figure 4.6). We propose three possible reasons for this: (1.) We saw a large degree of variation in the data, as indicated by the large errors. Using a higher dose of OA (as discussed in the previous section) might have led to a more robust effect with less variation in the data. In this regard, Sariahmetoglu and colleagues (2012) recently reported that 10 minutes of OA administration at 10 times the concentration we used (100 nM) before and after a period of 20 minutes GI exerted a cardioprotective effect in terms of functional recovery. (2.) In view of our hypothesis explaining the lack of an infarct sparing effect in hearts treated with OA at reperfusion alone or in combination with pretreatment (explained in the previous section), as well as the observation that the infarct limiting effects of pretreatment was not associated with functional recovery in the RI model, it seems probable that PP2A inhibition does not exert beneficial effects on the proteins involved in Ca^{2+} homeostasis. (3.) Studies comparing GI and RI (Lochner *et al.,* 2003; Bibli *et al.*, 2012) indicate that GI is a much more rigorous and stressful intervention than RI, rendering it more difficult to demonstrate changes in functional recovery due to an experimental intervention. In this regard, Lochner and colleagues (2003) concluded that IFS following RI is an easier and more reliable endpoint than functional recovery.

Despite the lack of functional recovery associated with OA treatment in this model, we decided to still use it to investigate the effects of OA treatment on the protein profiles of PP2A and other signalling proteins. The rationale behind this decision was that in order to assess the effects of OA on the cellular response to ischaemia and reperfusion a whole heart exposed to GI would be just as suitable as the area at risk in a heart exposed to RI, with the only difference that a GI intervention would generate more tissue for analysis than RI.

Effects of PP2A inhibition on protein profiles

As has already been alluded to in Chapter 1, there are numerous possible substrates for PP2A which could be of importance in the setting of I/R, ranging from apoptotic mediators to regulators of Ca^{2+} homeostasis. We decided to focus on the contribution of PP2A to the signalling dynamics associated with this setting, especially in view of the potential importance of PP2A as a regulator of signalling and bearing in mind that very little has been done in this regard. We also selected to specifically investigate the well-characterized RISK pathway (encompassing ERK p42/p44, as well as PKB/Akt and GSK-3β), as well as the much investigated, but still controversial p38 MAPK. These kinases were selected because they have been identified as key role-players in the balance between life and death.

When interpreting results generated by phosphatase inhibition, there is a simple golden principle which is often neglected, but should not be forgotten. This principle is simply that phosphatase inhibition in itself cannot stimulate or induce an increase in the phosphorylation of a substrate. It can only be associated with such an increased phosphorylation if the relevant opposing phosphorylation-stimulator (a kinase or activated receptor) is also active. In the absence of phosphorylating events (i.e. kinases), phosphatase inhibition is ineffectual and will have no visible consequences.

The effects of OA pretreatment on PP2A itself will be discussed first.

Protein phosphatase 2A-C

The administration of OA to our isolated rat heart model prior to global ischaemia, elicited changes in both posttranslational modifications of PP2A-C, namely methylation and phosphorylation.

The effect of OA administration on PP2A-C methylation

At the end of ischaemia, before the onset of reperfusion, OA treatment was associated with a significant increase in the absolute nonmethylation signal relative to control alone (Figure 4.8), implying that it was extending the effects of ischaemia alone on PP2A-C methylation. This effect became more robust during reperfusion and at 5 and 10 minutes reperfusion OA treatment was associated with an increase in nonmethylated PP2A-C relative to the whole population of PP2A-C (Figures 4.15 and 4.22). OA therefore favours the demethylation of PP2A-C, irrespective of ischaemia and reperfusion.

This is not an unexpected result, since although OA treatment is primarily associated with PP2A inhibition, it has been reported that OA also favours demethylation of PP2A-C (Favre *et al.*, 1997; Zhou *et al.*, 2008). In 1994 Floer and colleagues reported that OA treatment at concentrations which inhibit PP2A activity, favours demethylation of PP2A. Conversely, OA (40 nM) has been

reported to inhibit methylation of PP2A-C (Li *et al.*, 1994). This inhibition was found to be dependent on the concentration of PP2A-C present in the mixture and independent of the concentration of methyltransferase (in retrospect probably LCMT-1); implying that OA elicits its inhibitory effect via a direct interaction with PP2A-C, and not LCMT-1. Recently Stanevich *et al*. (2011) also reported that OA inhibits the LCMT-1 mediated methylation of PP2A-C.

Seemingly in contrast to this, in 1996 Lee *et al*. (1996) identified a methylesterase in bovine brain samples which specifically demethylates PP2A-C (in retrospect probably PME-1) and which is inhibited by OA at doses in the nanomolar range. Similarly, Ogris and colleagues (1999) reported that OA inhibited PME-1. Thus, OA can also inhibit the demethylation of PP2A-C.

These studies all together seem to confirm the original hypothesis put forward by Lee *et al.* (1996) and Li *et al.* (1998) that OA binding to PP2A-C blocks the accessibility of PP2A-C for both PME-1 and LCMT-1. If both enzymes are however blocked equally, one would expect no changes in the methylation of PP2A in the presence of OA, at least at saturation concentrations. The fact that OA has been found to either enhance or suppress methylation implies that differences in OA concentration, as well as the cellular milieu (or reaction mixture) to which the OA is added, will influence the eventual effect on methylation. In this regard it is interesting that Kranias *et al.* (2010) reported that in mast cells OA at lower concentrations (less than 100 nM) favoured methylation, reaching a peak of methylation at 100 nM, while at higher concentrations it seemed to suppress sustained methylation.

In our model, OA enhances the demethylation associated with ischaemia and reperfusion. As discussed in Chapter 3, we hypothesise that I/R is associated with an increase in the interaction between PME-1 and PP2A-C, thereby explaining the demethylation we observed at 20 minutes GI, as well as at 5 and 10 minutes reperfusion. Taking this into consideration, it seems that OA binding to PP2A-C in our model, for some unknown reason, blocks access to LCMT-1 specifically, thereby enhancing the demethylating effects of PME-1.

These observations emphasise the complexities associated with OA treatment, particularly in the scenario of I/R. Keeping in mind that methylation determines interaction with the regulatory subunits, thereby contributing to the regulation of substrate specificity and subcellular localisation, the question arises what the implications of OA treatment are on B subunit stability, as well as on substrate and intracellular targeting immediately following OA dissociation from PP2A-C.

The effect of OA administration on PP2A-C phosphorylation

In their original description of the tyrosine 307 phosphorylation of PP2A-C, Chen and colleagues (1992) reported that the autodephosphorylation of PP2A-C could be inhibited by OA, thereby

favouring enhanced phosphorylation of the enzyme. More recently Zhou *et al*. (2008) also reported that OA treatment favours PP2A phosphorylation.

Our data also confirmed these observations in that OA treatment increased the absolute level of phosphorylation of PP2A-C in comparison to control at 20 minutes GI (Figure 4.8) and exerted an even stronger effect at 5 minutes reperfusion, when OA administration increased absolute phosphorylation in comparison to 5 minutes reperfusion (without OA) as well, and also increased the degree of phosphorylation relative to total PP2A-C in comparison to control (Figure 4.15). Since 5 minutes reperfusion increased the phosphorylation of PP2A-C *per se* (Chapter 3), OA treatment simply enhanced this by inhibiting autodephosphorylation. It is also important to note that at 10 minutes reperfusion OA exerted no further effect on PP2A-C phosphorylation (Figure 4.22).

Our results imply that at especially 5 minutes reperfusion, under normal conditions, there is a large degree of autodephosphorylation exerted by PP2A, thereby maintaining PP2A activity at this time, in opposition to the concurrent increase in phosphorylation of PP2A-C which we have previously reported (Chapter 3). Autodephosphorylation therefore does not abolish the I/R mediated increase in PP2A-C phosphorylation (Chapter 3). By 10 minutes reperfusion, the effects of OA had dissappeared. This could be simply due to the effects of OA waning over time, although our methylation data would argue against this. Alternatively, the kinases involved in the phosphorylation of PP2A-C could be too dominant at this timepoint for our relatively small dose of OA to effectively/visibly influence phosphorylation/dephosphorylation. It could also be that the autodephosphorylating activity of PP2A is somehow downregulated at this time, possibly in association with the “storage” of inactive PP2A through interaction with PME-1. In all likelihood it is probably a combination of these three factors.

In summary: 20 minutes of GI and especially 5 minutes of reperfusion is associated with autodephosphorylation of PP2A-C opposing the I/R induced phosphorylation of this enzyme. Thus, even the activity of PP2A-C is in the end the product of both phosphatase (in the form of autodephosphorylation) and tyrosine kinase activity.

The effect of OA administration on PP2A holoenzyme assembly

Immunoprecipitation of PP2A-C from lysates of hearts collected at 5 minutes reperfusion, in the presence and absence of a pre-ischaemic administration of OA revealed an unexpected finding in that OA treatment was associated with a significant reduction in the physical interaction between PP2A-C and PP2A-A (Figure 4.29).

I am not aware of any previous work which has been done with OA which has investigated the effects of OA administration on dimer assembly. These results are however difficult to explain for

two reasons: First, Xing and colleagues (2006) managed to characterize the crystal structure of PP2A core enzyme bound to either OA or microcystin-LR. This implies that OA does not interfere with the formation of the core enzyme. Second, some authors have found that long-term treatment with OA is associated with an increase in the levels of PP2A-C (Favre *et al*., 1997; Zhou *et al.*, 2008). You would however not expect such an increase if PP2A-C was indeed dissociated from PP2A-A, since unless PP2A-C binds to another protein (such as PME-1 or α4), it will become unstabe and be degraded (Strack *et al.*, 2004; Van Kanegan *et al.*, 2005).

However, this observed OA mediated disassembly of the PP2A core enzyme is an intriguing result which, if confirmed, warrants further investigation.

Protein kinase B (Akt)

At 20 minutes GI, PKB/Akt is not phosphorylated, as can be seen from the relevant blot in Figure 4.9, in comparison to the positive control (a heart which was exposed to 20 minutes GI followed by 30 minutes reperfusion). This has also been observed by others (Mockridge *et al.*, 2000; Beauloye *et al.*, 2001). In keeping with the golden principle stipulated at the onset of this discussion, it is therefore not surprising that OA inhibition exerted no effect on PKB/Akt phosphorylation at this stage. Reperfusion however rapidly induced an increase in the phosphorylation of PKB/Akt (Figure 4.16). In this milieu of PKB/Akt phosporylation at 5 minutes reperfusion, PP2A inhibition was associated with an increase in the phosphorylation of PKB/Akt in comparison to control (Figure 4.16), suggesting that OA enhances the reperfusion mediated phosphorylation of PKB/Akt. At 10 minutes reperfusion phosporylated PKB/Akt is significantly elevated in both 10 minutes reperfusion *per se* and PreOA (Figure 4.23). This loss of OA specific effect could be due to either washout of OA at this time point, or it could be indicative of diminished importance of PP2A at this time.

From the data which we have, it is clear that PP2A dephosphorylates PKB/Akt within the first minutes of reperfusion; an observation which fits with the PP2A-PKB/Akt interactions which have been noted by others (Andjelković *et al*., 1996; Van Kanegan *et al.*, 2005; Zuluaga *et al.*, 2006; Mott *et al.*, 2008). Our co-immunoprecipitation experiments not only showed that PP2A is physically associated with PKB/Akt, but it also showed a strong trend for OA interfering with this association (Figure 4.30). The data is however unfortunately not definitive, although in combination with what is known in the literature it seems very probable that PP2A directly dephosphorylates PKB/Akt at 5 minutes reperfusion.

Glycogen synthase kinase-3β

Although GSK-3β has been reported to be downstream of PKB/Akt (Cross *et al.*, 1997), we already observed a dissociation between these kinases at 20 minutes GI, since GSK-3β was phosphorylated at this time (Figure 4.10). Pre-ischaemic OA administration enhanced the

phosphorylation of GSK-3β at 20 minutes ischaemia as evidenced by the significant difference between PreOA and control at this time (Figure 4.10). This OA-mediated enhancement of GSK-3β phosphorylation became more evident at 5 minutes reperfusion (Figure 4.17), but dissipated at 10 minutes reperfusion (Figure 4.24).

Our experiments therefore implicate PP2A as a phosphatase which targets GSK-3β both at the end of ischaemia, as well as within the first minutes of reperfusion. Since PKB/Akt is not activated during ischaemia, and therefore also not influenced by the presence of OA, the OA mediated elevation in the phosphorylation of GSK-3β which we observed must either be due to an interaction between PP2A and another upstream regulator of GSK-3β which is active during ischaemia, or PP2A must be directly dephosphorylating GSK-3β. Unfortunately we did not investigate either of these possibilities. Co-immunoprecipitation experiments at 5 minutes reperfusion however indicated a physical association between PP2A-C and GSK-3β (Figure 4.31), which was not influenced by OA administration. This would favour the model that PP2A inhibition does not influence GSK-3β directly, but rather exerts an effect on upstream signalling. This at least seems to be the case at 5 minutes reperfusion, and is very conceivable given the fact that OA favoured the phosphorylation of PKB/Akt at this time.

The question of the nature of the interaction between PP2A and GSK-3β at 20 minutes GI remains open. Our co-immunoprecipitation data at 5 minutes reperfusion both confirms the possibility of a direct interaction between GSK-3β and PP2A, but also casts some doubt on the idea since it actually implied that OA exerts its effects on GSK-3β at reperfusion via the activation of PKB/Akt. As discussed by Juhaszova and colleagues (2004) numerous pathways potentially signal on to GSK-3β. The main thrust of research in the field of I/R injury and cardioprotection has been aimed at elucidating cellular events either during or following and IPC stimulus, or during reperfusion with remarkably little known concerning events during ischaemia itself. It is therefore a matter of speculation concerning which potential kinases are activated during ischaemia and are responsible for the phosphorylation of GSK-3β. Potential kinases which have been implicated as upstream inhibitors of GSK-3β and which are also activated by ischaemia include PKC (Prasad & Jones, 1992) and PKA (Krause *et al*., 1983; Zhang *et al*., 2010). Of these two it is especially PKA which could potentially play a role during ischaemia, given the fact that sustained ischaemia is associated with an increase in cellular levels of cAMP (Rabinowitz *et al*., 1975; Krause *et al*., 1983; Zhang *et al*., 2010). If it is indeed PKA which is involved as an upstream inhibitor of GSK-3β during ischaemia, it is just as conceivable that the effects of OA are mediated through an increase in PKA activation, since PP2A has been implicated in the dephosphorylation of PKA as well (Liauw & Steinberg, 1996).

It must also be mentioned that there is not consensus regarding ischaemia induced phosphorylation of GSK-3β. In a hepatic model of I/R, Ren and colleagues (2011) found that 90 minutes of ischaemia actually slighly reduced GSK-3β phosphorylation. Obviously, differences in the organ investigated may explain this contrast between their data and ours.

We can therefore conclude that GSK-3β phosphorylation at the end of ischaemia and at the onset of reperfusion is sensitive to PP2A activity. Whether this happens through a direct interaction between GSK-3β and PP2A, or through the PP2A-mediated regulation of upstream GSK-3β kinases remains to be established. Our co-immunoprecipitation data at 5 minutes reperfusion favours the theory that OA exerts it effects upstream from GSK-3β.

p38 Mitogen activated protein kinase

As elaborated on in the introduction of this chapter, p38 MAPK is controversial, not only concerning its role in I/R and cardioprotection, but also with regards to the way in which it interacts with PP2A: either as a regulator or as a substrate.

At the end of ischaemia OA administration exerted no effect on the ischaemia induced phosphorylation of p38 MAPK (Figure 4.11). Similarly, PP2A inhibition had no significant effect on p38 phosphorylation at 5 or 10 minutes reperfusion (Figures 4.18 and 25). This then implies that in our model p38 MAPK is not a substrate for PP2A. Given this it was quite surprising that our co-immunoprecipitation experiments on tissue collected at 5 minutes reperfusion showed an association between PP2A and p38 MAPK which was mitigated by OA treatment (Figure 4.32). Following on the proposal discussed above, that OA binding to PP2A-C interferes with PME-1 and LCMT-1 binding to PP2A-C, it could be argued that OA binding to PP2A-C blocks an association with p38 MAPK. In this regard it has been shown that p38 MAPK can influence PP2A activity by physically associating with the enzyme (Grethe & Pörn-Ares, 2006).

Our data therefore favours a model where, in the setting of both ischaemia and reperfusion, p38 MAPK is upstream of PP2A and possibly contributes to its regulation by physically interacting with it.

Extracellular signal-regulated kinase p42/p44

We found that ischaemia suppressed ERK p42/p44 phosphorylation below control levels (Figure 4.12). The fact that OA administration failed to influence this indicates that PP2A is not involved in this ischaemia mediated dephosphorylation of ERK p42/p44. The question therefore remains open as to which phosphatase is involved in this response?

Intriguingly, PP2A is recruited as an ERK p42/p44 phosphatase at the onset of reperfusion, as shown by the profound increase in phosphorylation of ERK p42/p44 in OA treated hearts at 5 minutes reperfusion (Figure 4.19). This OA mediated effect was however lost at 10 minutes reperfusion (Figure 4.26). The contribution of PP2A to the dephosphorylation of ERK p42/p44 is further confirmed by our co-immunoprecipitation experiments which indicated an association between ERK p42/p44 and PP2A-C, which was abolished by OA treatment.

As discussed in the introduction, PP2A has been implicated as a positive regulator of the ERK p42/p44 pathway by contributing to activation of the upstream kinase Raf-1(Jaumot & Hancock, 2001; Abraham *et al.*, 2001; Adams *et al.*, 2005). Our data is however in agreement with the body of work which idenifies PP2A as an ERK p42/p44 phosphatase (Sontag *et al.*, 1993; Alessi *et al.*, 1995; Quintalje *et al.*, 1996; Nyunoya *et al.*, 2005) at the onset of reperfusion, but not at the end of ischaemia.

Summary

Having discussed the effects of PP2A inhibition on PP2A, the RISK pathway and p38 MAPK at the end of 20 minutes GI and the onset of reperfusion, some trends and issues have emerged which require further discussion:

1.) Although OA was administered immediately prior to sustained ischaemia it did not exert an effect on the selected kinases during ischaemia itself. The only exception to this is GSK-3β which showed enhanced phosphorylation in the presence of OA.

2.) Five minutes reperfusion is a very dynamic time point concerning the phosphorylation and dephosphorytion of the pro-survival kinases. It is at this time that PP2A shows the most outspoken autodephosphorylation, and that the effects of PP2A inhibition is most evident.

3.) The observations referred to in the previous paragraph were made using Western blotting techniques. We also applied a co-immunoprecipitation approach to further confirm and characterize these observations. On the one hand, this seems to have worked, since we found that in almost all instances (except for GSK-3β) OA treatment interfered with the interaction between these kinases and PP2A. On the other hand it is worrisome that 5 minutes reperfusion in itself was never associated with the expected increase in PP2A-kinase interactions relative to control. One possible explanation for this pertains to the way in which the lysates were prepared, and is based on the idea that different cellular populations of kinases and phosphatases are involved in different mechanisms. In order to co-immunoprecipitate we had to adjust our lysis buffer in order to avoid protein-protein dissociation. One of these adaptations involved the reduction of the concentration detergent in the buffer from 1% to 0.1% Triton X-100. This led to a better co-immunoprecipitation, but also

to less efficient extraction of proteins from the cell membranes. The implication of this is that by reducing the protein extraction from the membrane fraction, we inadvertently changed the composition of the lysate analysed, relative to the lysates used for the Western blots which were prepared in 1% Triton X-100. This opens the door for the possibility that, although the co-immunoprecipitation experiments revealed the impact of OA exposure in protein interactions, it did not include the same population of kinases as the lysates analysed by Western blotting, thereby possibly missing (or at least partially missing) those kinases of which the phosphorylation was influenced by the presence of OA.

Another adjustment that we had to make was the omission of PSP inhibitors (β-glycerophosphate and tetrasodiumpyrophosphate) from the immunoprecipitation lysis buffer. We did this to avoid possible interference in the interactions between PP2A and its substrates. In the case of the OA treated group OA had already bound to and inhibited PP2A in the cardiomyocytes, thereby interfering with the interaction between PP2A and its substrates. In the control and 5 minutes reperfusion group though, PP2A was not inhibited by anything – not even phosphatase inhibitors in the lysis buffer. This opens the door for the possibility of PP2A interacting with any potential substrate in the lysate mixture itself, sometime between the making of the lysate and preparing the final boiled sample containing Laemmli buffer. This would then in effect “mask” any differential intracellular association between PP2A and its substrates in the controls and at 5 minutes reperfusion alone.

These are limitations which we encountered while attempting co-immunoprecipitation with PP2A-C and necessitate the interpretation of this data in the context of the more conservative Western blotting data.

4.) Although we identified 5 minutes reperfusion as a dynamic protein phosphorylation-dephosphorylation timepoint, 10 minutes failed to show the same dynamic profile. There are three possible explanations for this: (1.) The one explanation which almost immediately comes to mind is that all the intracellular signalling processes which we measured have normalized at 10 minutes reperfusion. This conclusion is however only valid if enough OA is still present inside the myocytes to effectivley inhibit PP2A, and should ideally be tested by comparing the PreOA group with a control group which received perfusion throughout, in conjunction with a comparable OA administration (in order to assess the effects of OA on non-ischaemic control hearts). (2.) While characterizing the phosphorylation mediated inactivation of PP2A, Chen and colleagues (1992) postulated that the autodephosphorylation of PP2A presents a mechanism controlling the transient inhibition of PP2A, thereby opening a window of opportunity for signalling processes to happen uninhibited by PP2A. In our experimental setup such a “window of PP2A inhibition” would be characterized by an increased phosphorylation of

the enzyme, lack of OA-mediated changes to the phosphorylation of both PP2A and its substrates and strong phosphorylation of the involved signalling pathways. Ten minutes reperfusion seem to meet all these criteria, and as such could indicate an early endogenous mechanism to reduce PP2A activity in order to allow pro-survival signalling to occur unabated. (3.) Another possible, less glamorous but probably more likely, explanation is that the low OA dose initially administered has been washed out of the hearts by 10 minutes reperfusion. Our experimental design unfortunately does not allow us to determine which of these explanations are valid. The 10 minute reperfusion data should therefore be interpreted with great caution.

In conclusion, although PP2A shows significant phosphorylation (and therefore inhibition) at 5 minutes reperfusion, this is balanced by an active autodephosphorylation activity of the enzyme. We have identified PP2A as an important direct phosphatase of both PKB/Akt and ERK p42/p44 at 5 minutes reperfusion, but not during sustained ischaemia. PP2A also favours the activation of GSK-3β during sustained ischaemia and early reperfusion, although this does not seem to be mediated by a direct interaction but rather through the action of PP2A on signalling molecules upstream of GSK-3β. At no point in I/R is p38 MAPK a substrate of PP2A, although we did find evidence for a physical association between the two, revealing the possibility of p38 MAPK mediating some of its effects via PP2A.

PP2A inhibition in the isolated rat heart exposed to I/R: limitations and future directions

As discussed at length earlier in this chapter, we utilized a relatively low concentration of OA for a relatively short period of time. The advantage of this approach was that all effects observed could be ascribed with a high degree of certainty to PP2A, but not PP1 inhibition. It is however very likely that we would have generated more robust data, especially concerning functional recovery and protein profiles had we used a higher dose of OA, possibly also for a longer period of time. In this regard it would be interesting to repeat these experiments but with an OA dose of 100 nM, administered for no less than 20 minutes at a time.

A caveat is warranted at this time. It has been found that at PP2A specific concentrations OA inhibits not only PP2A, but also the less prevalent protein phosphatases PP3, 4, 5 and 6 (Honkanen *et al*., 1994; Herzig & Neumann, 2000; Janssens & Goris, 2001; Dounay & Forsyth, 2002). The obvious implication of this is that some of the effects we have observed might be attributable to any of these less well described phosphatases. This is a fundamental shortcoming using OA, and as more research is done on these phosphatases the relative contribution of each to the effects associated with OA administration will hopefully become clearer. For now, we assume that most of the effects elicited by OA can be ascribed to PP2A.

It is therefore clear that a fundamental problem in this type of research is the ability to modulate PP2A activity. OA is a well-accepted and -characterized tool to investigate PP2A, it is however not the only one. It would be beneficial to compare our OA studies with other PP2A and PP1 inhibitors such as fostriecin and calyculin A in an attempt to better elucidate the role of PP2A (and PP1 for that matter) in I/R. Silencing of PP2A-C (or PP2A-A, which would eventually lead to a reduction in PP2A-C levels (Van Kanegan *et al.*, 2004)) is also an approach which can be used to modify PP2A activity. It offers the advantage of greater specificity, although its application is limited to a cell based system and it will exert its effects over a longer period of time, which could complicate matters, especially if compensatory mechanisms come into effect (as noted by Van Kanegan *et al*., 2004). The ideal study would therefore combine PP2A silencing with pharmacological inhibition to better describe the role of PP2A.

It is also very unfortunate that we were not able to measure PP2A activity. This is a shortcoming which must be addressed, since it will allow quality control on whichever intervention is chosen to modulate PP2A activity. At the onset of this study we attempted to measure PP2A activity by measuring the generation of free phosphates in tissue lysates pre-cleared of phosphates, but failed to optimize the technique in our model (see Chapter 2). Other approaches to measure PP2A activity will have to be explored. Concerning this study, the low dose of OA which we used almost guarantees that the effects observed, can be ascribed to PP2A inhibition.

We found that pre-ischaemic treatment of heart preparations with OA conferred protection. Although we then analysed timepoints at the end of ischaemia and early in reperfusion in order to investigate the mechanisms at work, it would probably be insightful to also analyse the effects of OA on the protein profiles immediately before the onset of ischaemia. It may also be useful to investigate 15 minutes reperfusion in order to better understand the sequence of events in reperfusion and also to assess if there are any longterm effects elicited by OA.

As explained in the discussion, we utilized a model of 20 minutes GI in order to shed light on the mechanisms involved in the infarct sparing effect of OA administration in a model of 35 minutes RI. The rationale was that we were interested in the effects of OA administration on I/R tissue *per se*. We limited the duration of GI to 20 minutes in order to also assess functional recovery. If however the focus is purely to delineate the singalling mechanisms involved in 35 minutes RI, it would possibly be better to use a model of 35 minutes GI.

As also mentioned in Chapter 3, our failure to investigate the regulatory B subunits was a conscious decision to simplify the study by first focussing on the most basic elements, namely the core enzyme of PP2A. Our results however necessitate further research into the specific regulatory

subunits which are involved in targeting PP2A to its different substrates during ischaemia and reperfusion.

Linking with this, we chose to focus our study on the impact of PP2A inhibition on signalling events. The lack of effect of PP2A inhibition on functional recovery and the body of work which has implicated PP2A in the regulation of proteins involved in Ca^{2+} homeostasis, however make a good argument to also investigate the effects of PP2A inhibition on Ca^{2+} homeostasis in the setting of I/R.

We found that p38 MAPK is possibly an upstream regulator of PP2A. Such a relationship between these two signalling molecules have been described by others and as such warrant investigation in our setting as well. To do this we will have to modulate the activity of p38 MAPK (either through pharmacological means, or by silencing the enzyme) and then assess the effect of this on PP2A in terms of activity, cellular location and substrate specificity.

Finally, we utilized a co-immuprecipitation approach to assess the binding partners of PP2A-C at the onset of reperfusion. This approach is potentially very useful and contributes to the interpretation of the Western blotting results. In this study however, we can only utilize our immunoprecipitation data to indicate possible relationships which should be investigated in the future, since our results still require confirmation. Confirmation of co-immunoprecipitation results entails the immunoprecipitation of each of the proposed binding partners of PP2A and then probing for PP2A-C in order to see if it co-precipitates with these proteins. This is however a major undertaking which did not fall within the range of this project. Other forms of confirmation which could also be pursued entail the use of immunofluerescence microscopy in order to assess co-localisation of PP2A-C with these kinases, or FRET (Fluorescence Resonance Energy Transfer) analysis.

Despite these limitations and shortcomings, this study serves as a preliminary report on the contribution of PP2A to the signalling events associated with myocardial I/R. It has revealed some expected, and some intriguing results which set the foundation for further studies.

PP2A inhibition in the isolated rat heart exposed to I/R: summary and conclusion

The pre-ischaemic treatment of isolated rat hearts with 10 nM OA was associated with an infarct sparing effect, which can most likely be ascribed to the favourable elevation in phosphorylation of the pro-survival RISK pathways at the onset of reperfusion. PP2A is therefore involved in the negative regulation of these pathways during early reperfusion. Inversely to this, PP2A is a positive

regulator of GSK-3β during ischaemia, with PP2A inhibition favouring the inhibition of GSK-3β, which can potentially also have cardioprotective consequences.

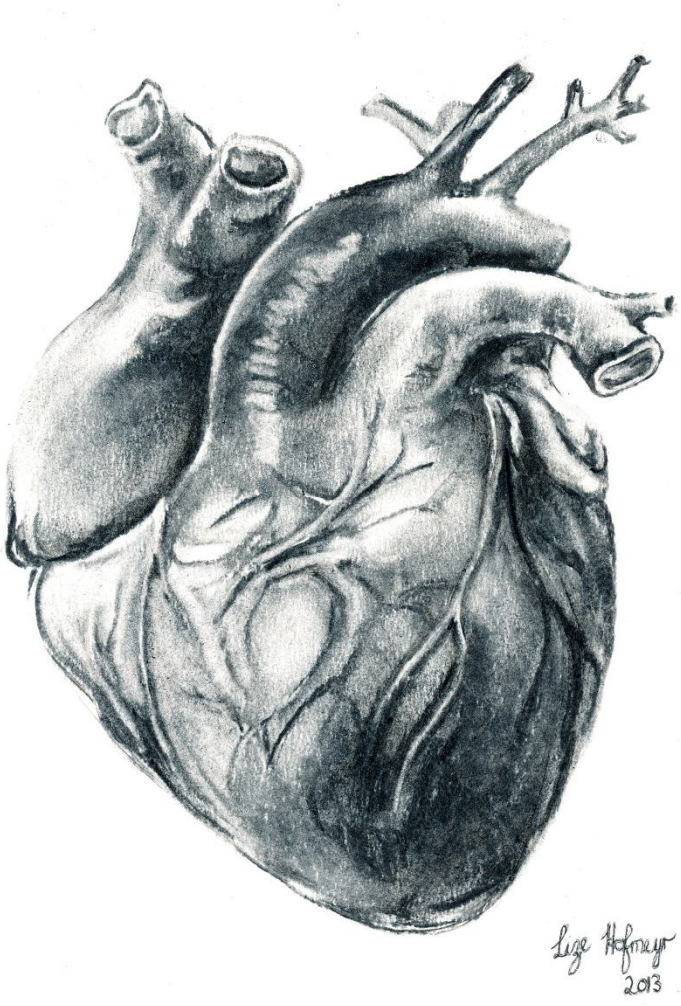
Our results have therefore placed PP2A as a relevant and important regulator of survival signalling during sustained ischaemia and especially at the onset of reperfusion.

CHAPTER 5

Pharmacological modulation of PP2A: Activation

"'Have you guessed the riddle yet?' the Hatter said, turning to Alice again.
'No, I give it up,' Alice replied. 'What's the answer?'
'I haven't the slightest idea,' said the Hatter.
'Nor I,' said the March Hare."

Lewis Carroll
Alice's adventures in wonderland



Chapter 5

Pharmacological modulation of PP2A: Activation

Introduction

The fungal species *Cordyceps* (class *Ascomycetes*, family *Cordycipitaceae*) was already recognised in Chinese folk medicine to have medicinal value when ingested. This traditional belief motivated modern studies into the composition of these fungi which, in turn, revealed several interesting fungal metabolites to medical science. One of the metabolites from *Isaria sinclairii*, myriocin, was especially interesting due to its immunosuppressive characteristics. In 1994, a novel compound was derived from myriocin which was called FTY720, also known as fingolimod (Chun & Brinkmann, 2011).

Although the initial interest in this drug centred on the possibility of utilizing its immunosuppressive abilities to combat transplanted organ rejection, it is its beneficial effects in the setting of multiple sclerosis (MS) which led to its eventual approval by the United States and European Union for clinical use as an oral treatment for MS under the commercial name Gilenya (Novartis) (Chun & Brinkmann, 2011; US Food and Drug Administration, accessed 2013).

Immunosuppressive mechanism in MS

MS is an autoimmune disease in which immune cells transverse the blood-brain barrier to attack the central nervous system, leading to eventual demyelination and neurodegeneration (Rubin, 2013). There are two mechanisms by which FTY720 can suppress this autoimmune response:

Regulating lymphocyte egress from the lymph nodes

The movement of lymphocytes from the lymph nodes in response to an immunological challenge is a regulated process characterized by an almost paradoxical initial retention of lymphocytes in the nodes in order to increase the number of antigen-specific T cells (Brinkmann, 2009). This retention is associated with a down-regulation of sphingosine-1-phosphate (S1P) receptor 1 (S1P1) in the T cells stationed in the lymph nodes, implicating S1P stimulated signalling in the regulation of T cell egress (Brinkmann, 2009). It is this S1P link to lymphoid function which supplies the point of interaction between FTY720 and the immune response.

FTY720 is a structural analogue of the sphingolipid, sphingosine, which is a structural component of cell membranes and of importance in cellular signalling (Hla & Brinkmann, 2011). Just like sphingosine, FTY720 easily crosses cellular membranes and can be phosphorylated intracellularly

by the enzyme sphingosine kinase 2 (SK2) (Karliner, 2009). Following its phosphorylation, phosphorylated FTY720 (P-FTY720) can exit the cell to act in a paracrine or autocrine fashion to bind to S1P receptors in the cell membrane. Of the five S1P receptors which have been identified, P-FTY720 can bind to S1P1, 3, 4 and 5 (Brinkmann, 2009).

Although P-FTY720 therefore acts as a S1P1 receptor agonist, it elicits its immunomodulatory effect by paradoxically acting, as Brinkmann (2009) puts it, as a "functional antagonist" of S1P signalling (Chun & Brinkmann, 2011). It elicits this effect by inducing the down regulation of S1P1 both in terms of receptor internalization, as well as promoting the proteosomal degradation of the receptor (Brinkmann, 2009). These antagonistic effects in the lymphoid tissue culminates in the suppression of especially central memory T cell egress from the lymph nodes (Hla & Brinkmann, 2011).

Effects of FTY720 on the central nervous system

Additional to these effects of FTY720 on the immune system, there is also some evidence for a direct protective interaction between FTY720 and the central nervous system. As described for the immune tissue, P-FTY720 down regulates S1P1 and S1P3 receptors in astrocytes in the central nervous system. This is beneficial, since both S1P1 and S1P3 have been implicated in mediating astrogliosis; the pathological transformation of astrocytes associated with several neurodegenerative conditions, such as MS (Brinkmann, 2009; Chun and Brinkmann, 2011).

In conclusion, FTY720 elicits its effects on MS by virtue of being a sphingosine analogue. It is phosphorylated to P-FTY720 which can then act as a S1P receptor agonist, but with the significant difference that it down-regulates the S1P receptors, thereby modulating the release of activated lymphocytes from the lymph nodes, as well as pathological transformation in the central nervous system. We were however not interested in FTY720 for its immunomodulatory effects. It is its potential as a tumour suppressor which is of more interest to us.

FTY720 as a tumour suppressor

FTY720 has also received some attention as a possible tumour suppressor, primarily through its effects on cell survival signalling via its modulation of the activity of the tumour suppressor PP2A.

Although PP2A has been implicated as an oncogenic effector, it has also been linked to tumour suppression (Arnold & Sears, 2008; Perrotti & Neviani, 2013). The combination of these two disparate characteristics can however be reconciled if one takes into account the multitude of PP2A holoenzymes which could potentially target a very wide variety of substrates in the cell (see the introductions to especially Chapters 3 and 4). There is therefore some interest in the potential clinical value of drugs which can activate PP2A, of which FTY720 is one (Perrotti & Neviani, 2013).

One of the first studies revealing this effect of FTY720 was published in 2003 by Matsuoka and colleagues. They reported that 45 minutes of exposure to 8 µM FTY720 activated PP2A, or a PP2A-like phosphatase, in Jurkat cells. They further confirmed this effect by illustrating that incubation of purified PP2A with 10 µM FTY720 was associated with an outspoken increase in phosphatase activity (175% increase in activity of a PP2A holo-enzyme and 120% increase of the PP2A core dimer). These latter results imply that FTY720 exerts a direct effect on PP2A activity. This direct effect of FTY720 on PP2A to increase its activity in cancerous cells in which PP2A activity was compromised, has since been demonstrated by several workers (Neviani *et al.*, 2007; Oaks *et al.*, 2013; Saddoughi *et al.* 2013).

FTY720 as an activator of PP2A

There seems to be two probable mechanisms by which FTY720 can induce an increase in the activity of PP2A: either directly, or through the activation of a signalling pathway.

Direct activation

Recently, Saddoughi and colleagues (2013) reported that FTY720 (5-10 µM) can directly bind to SET, a protein which has been identified as an endogenous inhibitor of PP2A, also known as I_2^{PP2A} (see the introduction to Chapter 3). Through this interaction FTY720 blocks the inhibitory interaction between SET and PP2A, in a mechanism akin to the way in which ceramide increases the activity of PP2A (Mukhophadyay *et al.*, 2009). This mechanism is dependent on the non-phosphorylated form of FTY720, with P-FTY720 showing no interaction with SET (Saddoughi *et al.*, 2013). It is also noteworthy that these authors determined phosphorylated PP2A as a measure of PP2A activity within this setting, where the phosphorylated form of the enzyme is inactive (see introduction to Chapter 3). This mechanism has two important implications concerning the cellular scenario within which it occurs, as well as the effects ascribed to FTY720.

First, the scenario within which it functions. Neviani and co-workers (2007) already made the observation that in some cancerous cell lines PP2A activity is suppressed due to an overexpression of SET. This means that FTY720 actually reactivates PP2A in these cancer cell lines (Saddoughi *et al.*, 2013), which might explain why FT720 can induce cell death in tumorigenic cells while not exerting any detrimental effects on normal body cells (Neviani *et al.*, 2007; Oaks *et al.*, 2013). This means that the effects of FTY720 on PP2A, at least through this mechanism, is limited to cells in which SET plays a substantial role in PP2A inhibition. It is however interesting that Matsuoka *et al.* (2003) showed a direct interaction between PP2A and FTY720 in a system containing purified PP2A, thereby implying the existence of some other SET independent mechanism. Confirming this possibility, Collison *et al.* (2013) used a nonphosphorylatable FTY720 analogue to activate PP2A in the setting of immunology. This implies that the analogue used elicited a direct effect (at least a S1P receptor independent effect) in a setting in which SET-

mediated PP2A inhibition is probably not relevant (although their work did implicate MID1 as a PP2A inhibitor, see the introduction to Chapter 3).

Second, concerning the experimental effects attributed to FTY720 administration. As mentioned previously, the immunomodulatory effect of FTY720 is actually mediated by the phosphorylated form of the drug which can act as a S1P analogue, thereby binding to S1P receptors in the cell membrane. We are therefore now confronted with a drug which can exert effects both in its unphosphorylated and phosphorylated form, a complex situation which recently confronted Oaks and colleagues (2013). They found that in myeloproliferative neoplasms, unphosphorylated FTY720 can activate PP2A through its interaction with SET. In contrast to this, P-FTY720 acting on S1P1, activates a signalling pathway which phosphorylates SET, thereby increasing its inhibitory function of PP2A, thus leading to the inhibition of PP2A. Despite FTY720 and P-FTY720 exerting these opposing effects on PP2A activity, treatment with FTY720 (10 mg/kg per day) improved survival in leukemic mice with no signs of toxicity. These results may imply that in their animal model of leukaemia, FTY720 exerted a predominantly PP2A-activating effect.

It however seems that P-FTY720, functioning as a S1P analogue and binding S1P receptors, does not necessarily always mediate a reduction in PP2A activity.

Activation through a signalling pathway

The family of S1P receptors are all G-coupled protein receptors which can activate a range of signalling kinases and cascades, including the RISK pathway, eNOS and others (Knapp, 2011; Igarashi & Michel, 2009). As such it is important to note that S1P and its associated signalling has been implicated in cardioprotection against I/R injury (Karliner, 2009; Egom *et al.*, 2010). One of the potential signalling proteins which has been implicated in S1P (and therefore also P-FTY720) signalling is Pak-1 (Ke *et al.*, 2012). There is however evidence that Pak-1 exerts most its effects via the activation of PP2A (Ke *et al.*, 2008; 2010; and 2012). That being said, it is very difficult to reconcile some of the seemingly opposing effects of P-FTY720 stimulation, since it has been associated with the simultaneous activation of Pak-1 and PKB/Akt (Egom *et al.*, 2010 and 2011). The most plausible explanation for the simultaneous activation of PP2A, as well as a probable substrate of PP2A (see Chapter 4), lies in the compartmentalization of signalling where PP2A and PKB/Akt are activated in different cellular locations.

It is therefore clear that the signalling cascades linked to P-FTY720 is more complex and extensive than the activation of PP2A alone.

Effects of FTY720 on tissues other than the heart

As already discussed, FTY720 in its phosphorylated form exerts immunomodulatory effects by binding to and desensitizing the S1P receptors, especially S1P1, thereby limiting the movement of lymphocytes from the lymph tissue into the circulation (Brinkmann, 2009; Hla & Brinkmann, 2011). This mechanism of immunosuppression differs from the classical immunosuppressors and allows FTY720 to exert very specific effects on the immune system without suppressing the responsiveness of the whole immune system (Chun & Brinkmann; 2011). Linking with the immuno-modulatory abilities of FTY720, Collison *et al.* (2013) found that FTY720 abrogates airway inflammation and the allergic response in mice exposed to house dust mite infection.

Its ability to activate PP2A has received special attention in cancer research, since the reactivation of PP2A in tumorigenic tissues in which PP2A is suppressed through overexpression of SET leads to a reduction in the activity of pro-survival signals such as ERK p42/p44 and PKB/Akt, thereby favouring apoptosis and cell death (Matsuoka *et al.*, 2003; Neviani *et al.*, 2007; Liu *et al.*, 2008; Perroti, 2013).

The fact that FTY720 in its phosphorylated state can bind to and activate the S1P receptors has also stimulated interest in the use of this drug in association with I/R, since S1P has been implicated in cardioprotection against I/R (Knapp, 2011; Somers, *et al.*, 2012; Karliner, 2013). In this regard Kaudel and colleagues have reported (in two separate publications in 2007) that FTY720 treatment is associated with increased survival in mouse models of *in vivo* renal and liver I/R. They administered 2 doses of FTY720 to mice exposed to liver ischaemia and reperfusion, with the first dose administered 30 minutes prior to ischaemia and the second dose given 5 minutes before reperfusion. Deviating slightly from this protocol they administered only one dose of FTY720 to mice exposed to renal ischaemia, 5 minutes prior to reperfusion. In both scenario's FTY720 was associated with increased survival, probably due to its anti-inflammatory effects, especially the depletion of T-cells in the peripheral blood.

Similarly Wei and co-workers (2010) reported that FTY720 administration reduced IFS and improved outcomes in a mouse model of transient middle cerebral artery occlusion. In this study FTY720 was also administered during ischaemia or reperfusion and exerted a neuroprotective effect also mediated rather by its anti-inflammatory effects than any direct effect on neuronal tissue.

Cardiovascular effects of FTY720

FTY720 has also been investigated in the context of the vasculature, as well as the heart.

The effects of sphingosine-1-phosphate and FTY720 on the vasculature

It seems that FTY720 exerts most of its effects on the vasculature by binding to and activating S1P receptors. This short section will therefore briefly discuss the effects of S1P stimulation on the vasculature as well.

The complexity of FTY720 action is enhanced by the different effects elicited by this drug, depending on which S1P receptor is stimulated, as well as which cell type is involved. Stimulation of the S1P2 and 3 receptors have been associated with vasoconstriction, while others have found S1P3 to exert a vasodilatory effect along with S1P1 (Karliner *et al.*, 2003; Means & Brown, 2009; Igarashi & Michel, 2009). Since S1P2 has a very low affinity for P-FTY720 (Brinkmann, 2009), it is only S1P1 and 3 which are relevant with regards to the effects elicited by P-FTY720.

Irrespective of whether it be S1P1 or S1P3 stimulation, in the vascular endothelium both seem to be able to elicit vasodilation by a PI3-kinase-PKB/Akt-eNOS pathway in conjunction with an increase in intracellular Ca^{2+} levels which alleviates the inhibitory interaction of caveolin with eNOS by mediating the replacement of caveolin with calmodulin. The end effect of these interrelated pathways is that eNOS is activated, leading to an increased production of NO which in turn stimulates vasodilation (Tolle *et al.*, 2005; Igarashi & Michel, 2009).

Vasoconstriction associated with S1P stimulation in vascular smooth muscle cells follows a classical smooth muscle contraction pathway involving an increase in intracellular Ca^{2+} combined with the stimulation of Rho-associated kinase (ROK) and specific PKC isoforms which inhibit the activity of myosin light chain phosphatase (MLCP). The net result is an increase in the phosphorylation of the myosin light chain which then leads to contraction (Brinkmann, 2007; Igarashi & Michel, 2009; Means & Brown, 2009).

The precise effects of FTY720 on the vasculature in humans is uncertain. Westhoff and colleagues (2007) found that FTY720 treatment of renal transplant recipients failed to show beneficial vasodilatory effects. In fact, replacing FTY720 with mycophenolate mofetil (MMF) improved arterial stiffness as well as flow-mediated dilation. These results therefore suggest that long-term FTY720-treatment predominantly exerts a vasoconstrictory effect in humans.

FTY720 and the heart

Heart tissue expresses S1P receptors 1, 2 and 3 (Brinkmann, 2007; Means & Brown, 2009; Hla & Brinkmann, 2011), of which only receptors 1 and 3 are relevant when considering the effects of

FTY720. Both these receptors are associated with a reduction in heart rate (Hla & Brinkmann, 2011). This seems to be due to the interaction between activated S1P receptors and the inwardly rectifying atrial K^+ channel (IKACh). Hofmann (2007) reports that activation of the S1P receptors activate IKACh in atrial myocytes thereby inducing an increase in the inward flux of K^+, resulting in the hyperpolarization of the cell membranes and a reduction in membrane excitability. Interestingly, Hofmann (2007) speculates that prolonged exposure of the heart to S1P or FTY720 stimulation leads to a desensitization of the receptors, allowing normalization of the heart rate. This transient FTY720-mediated reduction in heart rate has also been reported in humans (Schmouder *et al.*, 2012). In conjunction with its effects on heart rate, S1P1 activation also exerts a negative inotropic effect (Means & Brown, 2009) which is probably linked to the activation of IKACh, as well as G_i mediated reduction in cAMP levels (Means & Brown, 2009).

As already mentioned, S1P has been associated with cardioprotection against I/R injury (Knapp, 2011; Somers *et al.*, 2012; Karliner, 2013). It is therefore no surprise that FTY720 has also received attention in the context of myocardial I/R injury. Research which has been published concerning the potential cardioprotective abilities of FTY720 is summarized in table 5.1. The last column of the table highlights the controversies regarding the effects of FTY720. It seems there is relative consensus regarding the ability of FTY720 to mediate functional recovery after an I/R incident (Hofmann *et al.*, 2009; Vessey *et al.*, 2013). It is also evident that it exerts an important effect on the rhythmicity of the heart, although the exact nature of this is unknown. Egom and colleagues (2010) found that FTY720 reduced the occurrence of rhythmic disturbances following I/R. In stark contrast to this, Hofmann *et al.* (2010) reported that FTY720 administration at reperfusion increased mortality in an *in vivo* model due to an increased occurrence of dysrhythmias. Intriguingly, the administration of FTY720 both prior to ischaemia, as well as during reperfusion increased survival (Hofmann *et al.*, 2010). In conjunction with this increase in survival, pre-treatment with FTY720 24 hours prior to ischaemia also induced a reduction in S1P1 receptor expression. It therefore seems that in this setting FTY720 exerted a cardioprotective effect not by acting as a S1P receptor agonist, but rather by acting as a functional antagonist which stimulates the desensitization of the S1P1 receptor. Concerning the ability of FTY720 to preserve the viability of tissue exposed to I/R, the results are inconclusive with two of the studies reporting an inability for FTY720 to reduce IFS (Hofmann *et al.*, 2009 and 2010) while two other studies reported that FTY720 can protect against cell death (Egom *et al.*, 2011; Vessey *et al.*, 2013).

Potential reasons for these contradictory results are unknown, although differences in experimental models probably contribute to a large degree. The study which reported on the detrimental effects of FTY720 treatment on reperfusion rhythmicity (Hofmann *et al.*, 2010) was the only *in vivo* study, thereby including the systemic effects of FTY720 on inflammation and vascular tone. The rest of the *in vitro* studies differ with regards to dosage, mode of application, animal species used, type of

Table 5.1. Summary of studies that have been published concerning the effects of FTY720 administration on the development of myocardial I/R injury. No consensus emerges, with studies showing deleterious, as well as cardioprotective effects.

LVdevP: Left ventricular developed pressure; LVEDP: Left ventricular end-diastolic pressure.

Reference	Experimental model	End-point	FTY720	Administration of FTY720	Finding
Hofmann *et al.*, 2009	Isolated rat hearts exposed to 30 minutes GI and 90 minutes reperfusion.	IFS and functional recovery.	50 or 500 nM*	Administered at the onset of reperfusion.	500 nM increased LVdevP and reduced LVEDP. Failed to reduce IFS.
	Human myocardial muscle strip preparations exposed to 90 minutes SI and 120 minutes reperfusion.	Functional recovery.	1 µM*	Administered at the onset of reperfusion.	Improved recovery of developed pressure.
	Isolated neonatal rat cardiomyocytes exposed to 6 hours hypoxia followed by 2 hours reoxygenation.	Cell viability.	100 nM*	Either pre-ischaemic, or at the onset of reperfusion.	Failed to elicit any protection.
Hofmann *et al.*, 2010	*In vivo* model of 45 minutes RI and 24 hours reperfusion induced in a rat model.	IFS and survival.	0.5 mg / kg	Either before reperfusion only, or 24 hours before ischaemia and again before reperfusion.	Failed to reduce IFS. Combined pre- and post-ischaemic administration improved survival, while reperfusion administration alone was associated with significantly poorer survival due to an increased occurrence of tachycardias or ventricular fibrillation.
Egom *et al.*, 2010	*Ex vivo* rat heart and sinoatrial node models exposed to 20 minutes perfusion with ischaemic solution followed by 30 minutes normal perfusion.	Rhythm disturbances.	25 nM	Added during ischaemia and reperfusion.	Reduced the occurrence of rhythmic disturbances in both models.
Egom *et al.*, 2011	Isolated neonatal rat cardiomyocytes exposed to 24h of $CoCl_2$-induced hypoxia or 20 minutes of ischaemia-simulating medium.	Cell viability.	25 nM	Present during hypoxia or simulated ischaemia.	Increased cell viability following exposure to both hypoxia, as well as simulated ischaemia.
Vessey *et al.*, 2013	Isolated mouse hearts exposed to 50 minutes of GI and 40 minutes reperfusion.	Both functional recovery as well as IFS.	0.6 µM	As a postconditioning intervention of 4 cycles of 5 seconds I/R.	Increased LVdevP and reduced IFS.

*This study utilized a phosphorylated form of FTY720.

simulated ischaemia or hypoxia induced in the cellular models, etc. There is almost no common ground between these studies, illustrating some of the typical challenges in basic cardioprotection research.

It is also interesting to note that all of these studies focussed on the application of FTY720 during ischaemia and reperfusion. None of the studies investigated the effects of FTY720 treatment directly prior to sustained ischaemia. Despite the probable effects of FTY720 on PP2A activation, none of these studies investigated this link either. In this regard it is noteworthy that Egom *et al.* (2011) reported that FTY720 treatment exerted its effects via the activation of both PKB/Akt and Pak-1, the latter also being implicated by others as an upstream activator of PP2A (Ke *et al.*, 2008; 2010; and 2012).

FTY720 therefore exerts an effect on the heart in the context of I/R, although the precise nature of this still requires investigation. The question regarding the effects of FTY720 on the heart is of clinical interest for two reasons: First, since MS patients are using FTY720 it is useful to define the effects of FTY720, and secondly since FTY720 does not exert toxic effects (Neviani *et al.*, 2007; Oaks *et al.*, 2013), even at high doses, it is a very attractive drug for clinical use.

Summary

FTY720 is a sphingosine analogue, which can utilize the same cellular mechanisms as sphingosine to exert its effects, i.e. FTY720 can be phosphorylated and following this can bind to, and activate S1P receptors. Initial interest in FTY720 focussed on its immunosuppressive effects which were exploited for its clinical use in the management of MS. It has however also been shown that FTY720 exerts a cytotoxic effect on cancerous cells, mediated by the activation of PP2A. As a S1P analogue FTY720 also influences several aspects of cardiac function including vascular tone, contraction, rhythmicity, and resistance to I/R injury.

We are therefore confronted by an intriguing pharmacological drug which can exert a multitude of effects, both in its unphosphorylated as well as phosphorylated forms. Of these effects, it is especially its ability to activate PP2A which elicited our interest.

Motivation, hypothesis and aims

FTY720 has been implicated in the activation of PP2A, both directly in its unphosphorylated form, as well as via activation of a signalling pathway. We therefore decided to utilize FTY720 to manipulate PP2A activity in conjunction with I/R to better define the contribution of PP2A to the myocardial response to I/R. Comparison of inhibitor studies (Chapter 4) with activator studies (FTY720 mediated) will contribute greatly to delineating the role of PP2A in I/R.

The fact that FTY720 is already in clinical use opens up questions regarding its potential effects on myocardial I/R injury. Not much research has been done on this aspect of FTY720 and the work done thus far has delivered contradictory results (table 5.1), did not focus on PP2A *per se* and, very importantly, has not investigated the effects of FTY720 treatment directly prior to sustained ischaemia.

We therefore decided to investigate the effects of FTY720 treatment on the outcomes of /IR in our isolated working rat heart model, as well as the effects of the drug on intracellular signalling in this setting. More specifically we aimed to determine:

- the effects of FTY720 treatment (prior to ischaemia or during reperfusion) on IFS in a model of regional ischaemia, as well as functional recovery in a model of global ischaemia.
- the effects of FTY720 treatment on signalling (specifically the RISK pathway, p38 MAPK and PP2A) in the context of global ischaemia and reperfusion.

Due to the miscellaneous effects of FTY720 it is difficult to hypothesize on the outcomes. In view of the literature, we expect FTY720 to induce a degree of bradychardia as well as vasodilation (or constriction) in our hearts, which in turn, will probably influence the outcome of I/R injury. In its capacity as a S1P agonist, it is very likely that FTY720 will exert a protective effect. In Chapter 4 we however found that PP2A is a negative regulator of the RISK pathway at reperfusion. If this is the case, and if FTY720 does increase PP2A activity in our model, it is also possible that FTY720 given at reperfusion will be detrimental to hearts exposed to I/R. This activation of PP2A will induce a reduction in the phosphorylation of PKB/Akt, GSK-3β and ERK p42/p44.

Despite the uncertainty about the outcomes, the results generated should prove interesting, novel and thought-provoking.

Material and methods

Rationale

In Chapter 4 we described the effects of PP2A inhibition on isolated rat hearts exposed to I/R in an attempt to delineate the role of PP2A regarding the outcomes of I/R injury, as well as the signalling events associated with this. This part of the study aims to address the same question, but by approaching it from the opposite direction, i.e. determining the effects of PP2A activation on the outcomes of I/R, as well as investigating the signalling events associated with the activation of PP2A in this setting. We therefore utilized a similar approach as in chapter 4, as set out in Figure 5.1.

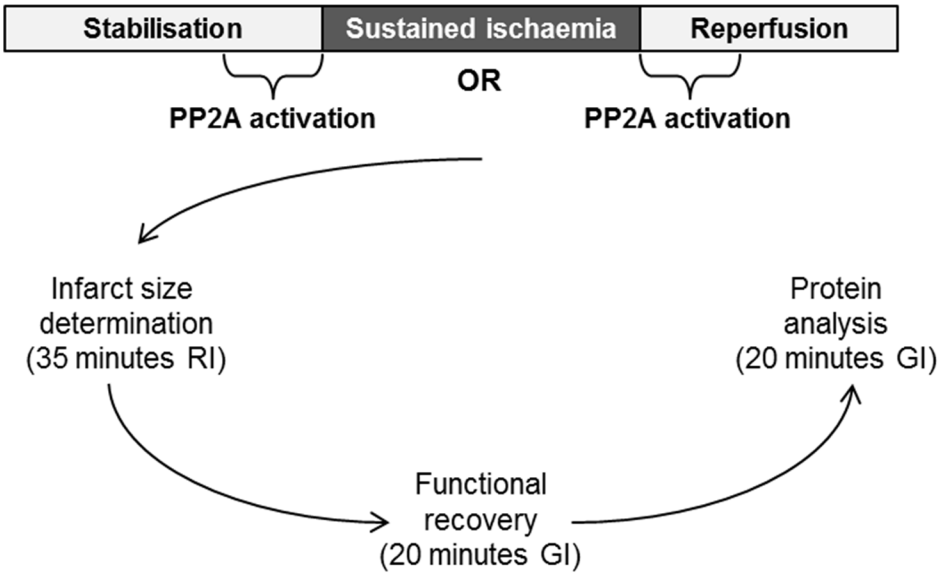


Figure 5.1. Schematic presentation of our approach to determine the effects of PP2A activation on the outcomes and signalling pathways associated with myocardial I/R.
We utilized IFS (following 35 minutes of RI) as our primary endpoint. Protocols which elicited a signifcant change in IFS were further assessed for their ability to influence functional recovery following 20 minutes GI, as well as to determine the protein profiles (PP2A, the RISK pathways and p38 MAPK) associated with these administration protocols.

Protocols

FTY720 dosage in the isolated perfused rat heart

FTY720 was obtained from Cayman Chemical (product number 10006292; Cayman Chemical, MI, USA). The drug was first dissolved in dimethyl sulfoxide (DMSO) and then added to the perfusate. In all studies the concentration of DMSO never exceeded 0.03% (vol/vol), i.e. 0.004 M. Since this concentration is well below the level of DMSO associated with changes in cardiac function (Bell *et al.*, 2008) or toxicity (Shlafer & Karow, 1975), and given that we found that up to 0.05% DMSO did not influence signalling in a model of RI (Van Vuuren, 2008), vehicle controls were not included in this study.

Several different dosages of FTY720 have been reported for isolated heart studies in the literature (Table 5.1). Based on these reported dosages we assessed three different concentrations of FTY720 (0.5, 1 and 2.5 µM) in terms of their ability to activate PP2A under baseline conditions. Measuring PP2A activity, as alluded to before, is itself a challenging exercise. We utilized two different approaches: (1.) Western blotting was used to determine the degree of PP2A phosphorylation, where phosphorylated PP2A is inactive (Chen *et al.*, 1992). Saddoughi and co-workers (2013) also used the same approach. (2.) The activity of PP2A immunoprecipitates was

measured directly, using a commercially available kit which utilized a substrate (DiFMUP) which is cleaved by protein phosphatases to generated a fluorescent product (DiFMU), as described in Chapter 2. Tissue samples for both these experimental approaches were prepared as shown in Figure 5.2. FTY720 was administered for a period of 15 minutes following a suitable time period of stabilisation and in the absence of any other experimental interventions.

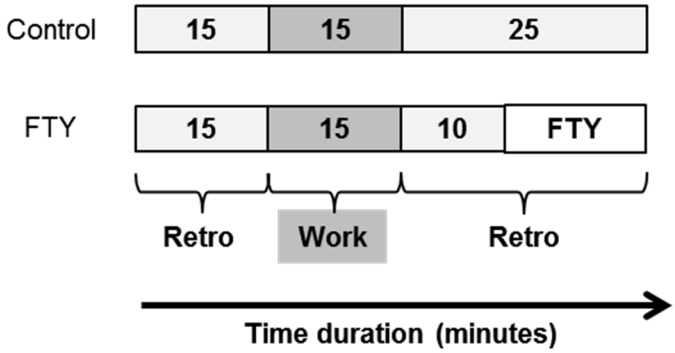


Figure 5.2. Determination of the best concentration of FTY720 to administer to the isolated rat heart in order to increase the activity of PP2A. Three different concentrations of FTY720 were tested: 0.5, 1 and 2.5 µM.

Infarct size (IFS)

The effects of FTY720 (1 µM and 2.5 µM) administration, both immediately before sustained ischaemia or at the onset of reperfusion (Figure 5.3), were evaluated. These two time points were chosen in order to match our inhibition studies (Chapter 3); to assess the effects of PP2A activation during both ischaemia, as well as reperfusion; and also to address the lack of data concerning the effects of FTY720 administered immediately prior to ischaemia.

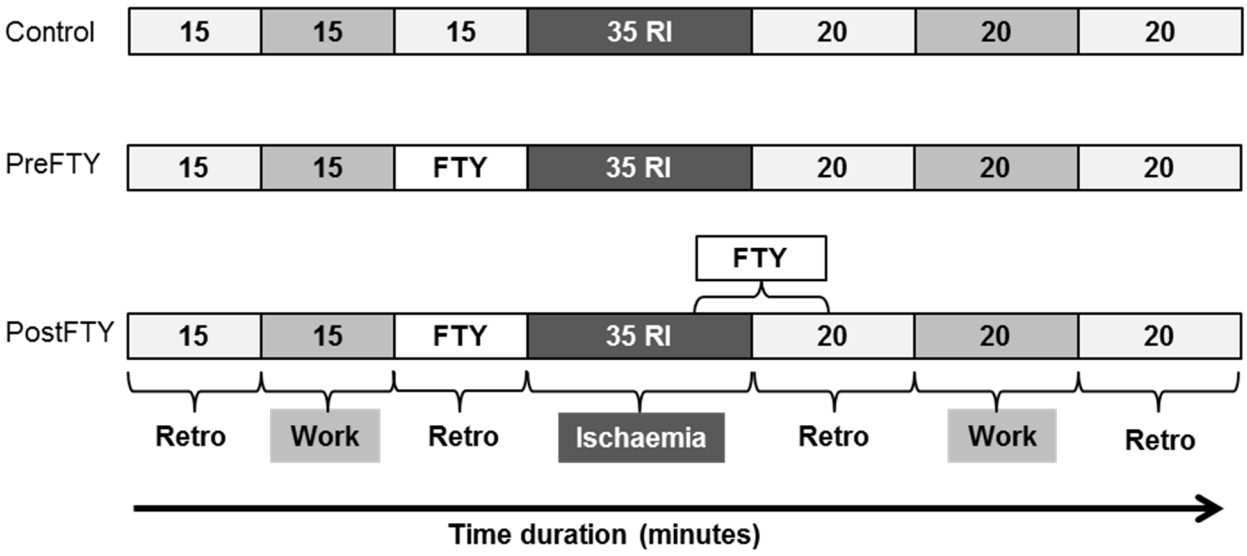


Figure 5.3. Protocols for determining the effects of FTY720 administration on infarct size. FTY720 was administered either directly prior to sustained ischaemia for a duration of 15 minutes, or at the very onset of reperfusion (PostFTY, for the final 5 minutes of RI and the first 10 minutes of reperfusion). Both protocols were followed for both 1 µM and 2.5 µM FTY720.

In addition to these studies, the effects of FTY720 administered as a preconditioning stimulus (Figure 5.4) were also determined. For this we specifically utilized 1 µM FTY720. For most of our

ex vivo experiments we administered FTY720 for a period of 15 minutes, the exception being the preconditioning administration of FTY720 which we limited to 10 minutes in order for it to fit into the general timing of the perfusion protocols (Figures 5.2, 5.3 and 5.4). As described in Chapters 2 and 4, 35 minutes of regional ischaemia was utilized in combination with 60 minutes of reperfusion.

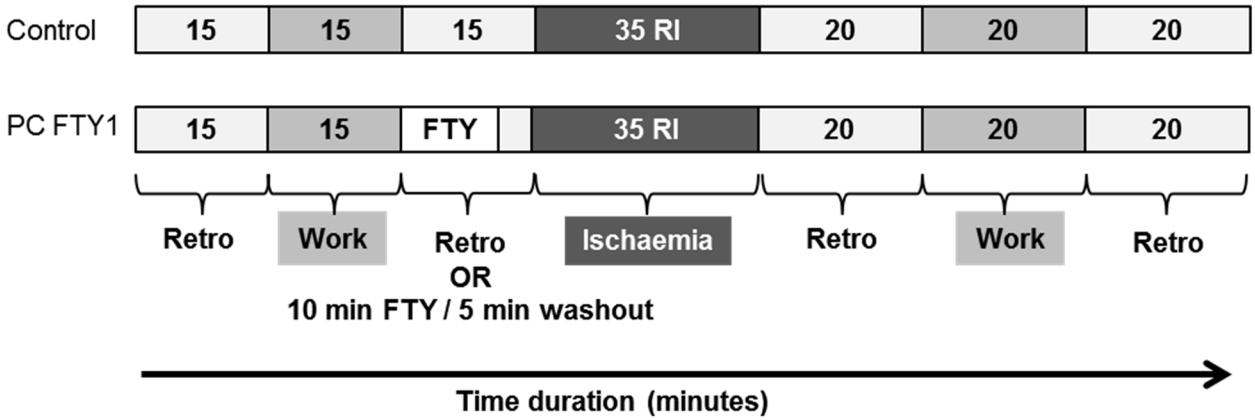


Figure 5.4. Protocol followed for preconditioning with FTY720. For this protocol we only utilized 1 µM of FTY720. FTY720 was administered for a period of 10 minutes, followed by 5 minutes reperfusion before the onset of sustained ischaemia.

Functional recovery

The effects of FT720 administration on functional recovery were studied during reperfusion following 20 minutes of global ischaemia. Three experimental protocols were employed in the setting of sustained global ischaemia (Figure 5.5).

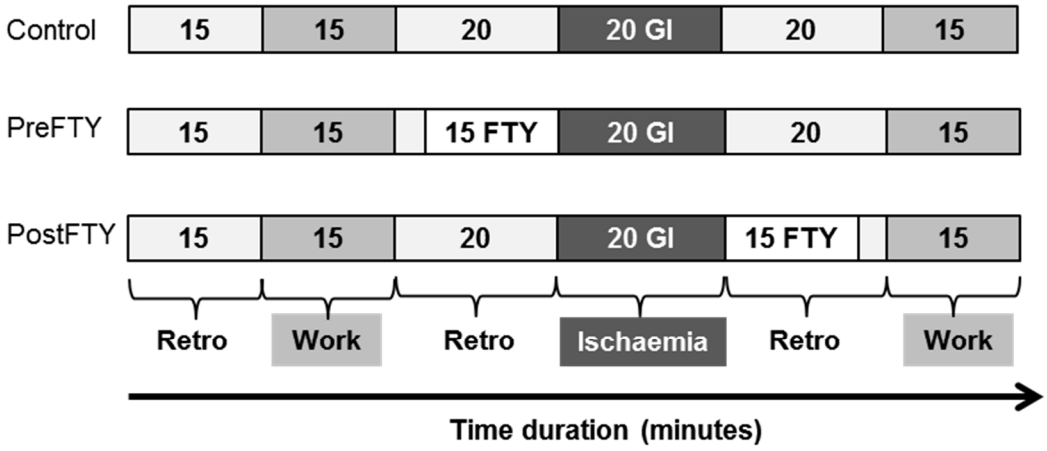


Figure 5.5. Protocols for determining the effects of FTY720 administration on functional recovery following 20 minutes GI.
FTY720 was administered either directly prior to sustained ischaemia for a duration of 15 minutes, or for the first 15 minutes of reperfusion. Both these administration protocols were followed for both 1 µM and 2.5 µM FTY720.

Protein profiles

Since the central focus of this project was to determine the role and importance of PP2A in the myocardial response to I/R, we decided to focus our experimental approach at this stage on a single FTY720 concentration. Since we found that FTY720 at a concentration of 1 µM, administered for 15 minutes, was adequate to increase PP2A activity (as will be reported in the next section), we only investigated the effects of this dose administered either prior to ischaemia or at the onset of reperfusion on mediators of the RISK pathway (ERK p42/p44, PKB/Akt and GSK-3β), p38 MAPK and PP2A. Different timepoints were investigated to assess the temporal spectrum of effects elicited by FTY720 on these signalling proteins (Figure 5.6).

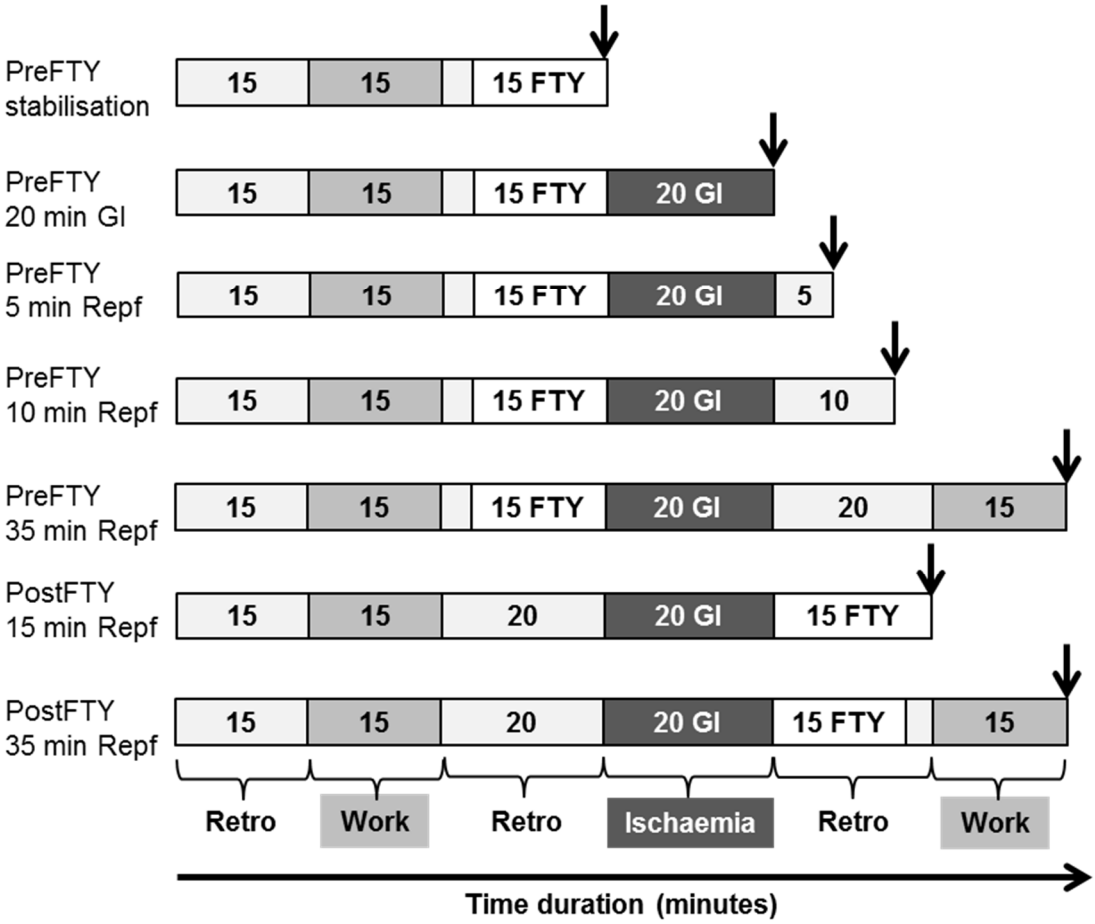


Figure 5.6. Protocols used to investigate the effect of FTY720 (1 µM) administration on protein profiles.
Hearts were freeze-clamped at different timepoints to investigate the effects of both pre-ischaemic, as well as reperfusion administration of FTY720 on PP2A, PKB/Akt, GSK-3β, ERK p42/p44 and p38 MAPK.

Statistical analysis

Multiple groups were compared with each other by performing an ANOVA analysis. Unless otherwise specified, the Bonferroni *post hoc* test was applied for all graphed data. Exceptions to this was the comparison of only two groups with each other, for which a T-test was used, or

analyses where multiple experimental interventions were compared with control alone, in which case Dunnett's test was applied.

Results

FTY720 dosage in the isolated perfused rat heart

We utilized two different approaches to determine which concentration of FTY720 (0.5, 1 or 2.5 µM) would increase the activity of PP2A under pre-ischaemic baseline conditions. Firstly, standard Western Blotting techniques were used to determine the effect of FTY720 on the phosphorylation of PP2A, since the phosphorylation of PP2A is associated with its inhibition (Chen *et al.,* 1992). Results are shown in Figure 5.7 (A-C). FTY720 treatment did not exert an effect on the levels of PP2A-C relative to control. All three concentrations FTY720 reduced the absolute phosphorylation of PP2A (Control: 1.00±0.11 AU vs. 0.5 FTY: 0.65±0.08 AU, 1 FTY720: 0.48±0.03 AU & 2.5 FTY: 0.62±0.04 AU, n=2-4; $P<0.05$). However only 1 µM and 2.5 µM of FTY720 were associated with a reduction in the phosphorylation of PP2A relative to total PP2A-C (Control: 1.00±0.14 AU vs. 1 FTY720: 0.57±0.04 AU & 2.5 FTY: 0.61±0.05 AU, n=2-4; $P<0.05$).

Secondly, PP2A activity was measured in PP2A-C targeted immunoprecipitates incubated with a phosphatase substrate (DiFMuP) which generates a fluorescent product (DiFMU) when hydrolyzed by a phosphatase (as described in Chapter 2). Okadaic acid (10 nM) was used to distinguish PP2A-mediated phosphatase activity even further. For this aspect of the evaluation of FTY720, we assayed tissue perfused with either 1 µM or 2.5 µM FTY720. As shown in Figure 5.7 (D) both these concentrations slightly, yet significantly, increased the activity of PP2A (Control: 400.8±23.75 fluorescence units vs. 1 FTY: 475.3±5.41 fluorescence units and 2.5 FTY: 489.0±20.11 fluorescence units, n=2-4; $P<0.05$).

Both these experimental approaches indicated that both 1 µM and 2.5 µM FTY720, when administered for 15 minutes, are sufficient to increase the activity of PP2A. To avoid non-PP2A related effects associated with FTY720 administration, we decided to use the lowest dose to investigate the effects of PP2A activation on the selected signalling molecules of interest. Since FTY720 is a clinically relevant drug, of which the effects in the setting of myocardial I/R are still very much uncertain, both concentrations were included in our initial study concerning the effects of FTY720 administration on the outcomes (IFS and functional recovery) of myocardial I/R.

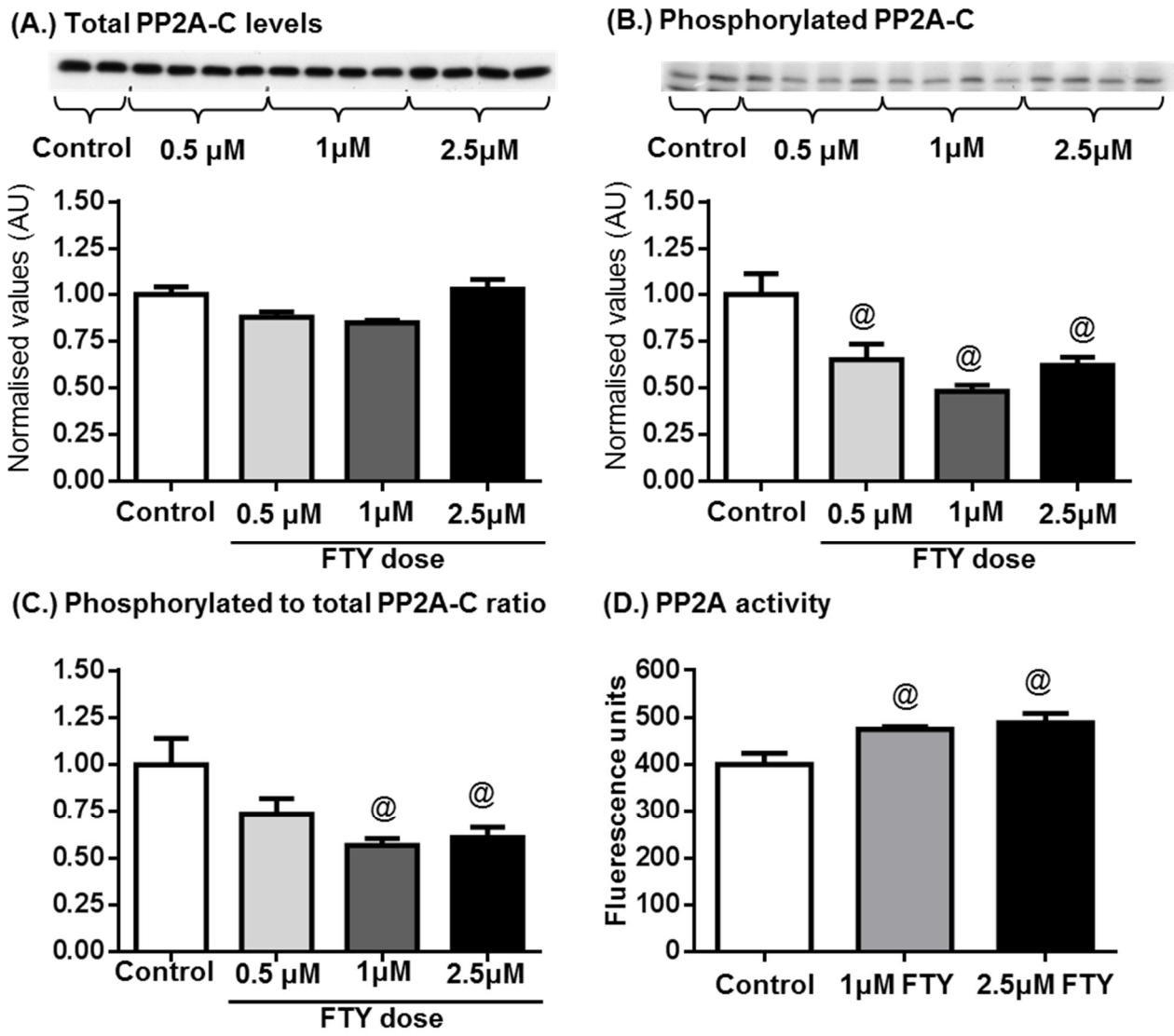


Figure 5.7. The effects of different doses FTY720 on PP2A activity.
Hearts were perfused for 15 minutes with different doses of FTY720 to determine the optimal concentration to elicit an increase in PP2A activity. FTY720 did not exert an effect on protein levels (A), but reduced the phosphorylation of PP2A-C, especially 1 µM and 2.5 µM FTY720 (B&C).
We proceeded to also determine the effects of FTY720 treatment on PP2A activity in the absence of any other experimental interventions (D). Both 1 µM and 2.5 µM of FTY720 increased PP2A activity.
Dunnett's post hoc test; @ P<0.05 vs. Control; n=2-4.

The effect of PP2A activation by the administration of FTY720 on infarct size

In Chapter 4 we utilized the pharmacological inhibition of PP2A either prior to ischaemia, or during the clinically relevant first moments of reperfusion in an attempt to better understand the contribution of PP2A to the development of I/R injury. Here we extend these experiments by activating PP2A activity at the same time points. Additional to these pre-ischaemic and reperfusion treatment based studies, we also included a preconditioning group in which 10 minutes of FTY720

(1 µM) was followed by 5 minutes washout before the onset of ischaemia. This intervention was included to better investigate the effects of FTY720 *per se* on IFS.

Baseline, pre-drug and -ischaemic functional parameters for all of these groups are shown in table 5.2. There were no significant differences between any of these groups and control (using Dunnett's correction).

Table 5.2. Pre-ischaemic functional parameters of isolated rat hearts exposed to 35 minutes regional ischaemia in conjunction with different administration protocols of FTY720.
**PC FTY1: Preconditioned with 1 µM FTY720.*
†Coronary flow measured during retrograde perfusion.

Group	Coronary flow (ml/min)†	Aortic output (ml/min)	Cardiac output (ml/min)	n-value
Control	9.00±0.52	48.33±3.56	62.83±4.34	6
PreFTY1	8.92±0.45	55.50±1.63	69.08±3.98	6
PostFTY1	10.00±0.82	44.00±4.30	58.14±5.49	7
PC FTY1*	8.51±0.35	40.57±2.21	53.64±3.03	7
PreFTY2.5	10.25±0.54	47.00±3.53	63.17±4.39	6
PostFTY2.5	9.67±0.76	52.33±3.20	69.42±3.94	6

For statistical comparison of IFS data we only compared different administration protocols of the same concentrations of FTY720.

The effect of 1 µM FTY720 on infarct size and associated functional recovery

The effects of the different protocols of FTY720 administration at 1 µM is shown in Figure 5.8. Reperfusion treatment with FTY720 reduced infarct size in comparison to control (Control: 39.89±3.93% vs. PostFTY: 23.96±3.99%, n=6-7; $P<0.05$), while pretreatment with the drug actually increased IFS (Control: 39.89±3.93% vs. PreFTY: 51.73±2.36%, n=6; T-test: $P<0.05$). This was an unexpected effect and prompted us to also investigate the effect of FTY720 preconditioning on IFS. Although preconditioning with FTY720 failed to reduce IFS relative to control (Control: 39.89±3.93% vs. PC FTY720: 35.58±4.73%, n=6; non-significant), it resulted in a lower IFS than pretreatment (PC FTY720: 35.58±4.73% vs. PretFTY: 51.73±2.36%, n=6-7; T-test: $P<0.05$). These data confirm the detrimental effects of FTY720 pretreatment immediately before sustained ischaemia, since a period of only 5 minutes washout between FTY720 administration and the onset of RI alleviated the FTY720-mediated increase in IFS observed with pretreatment.

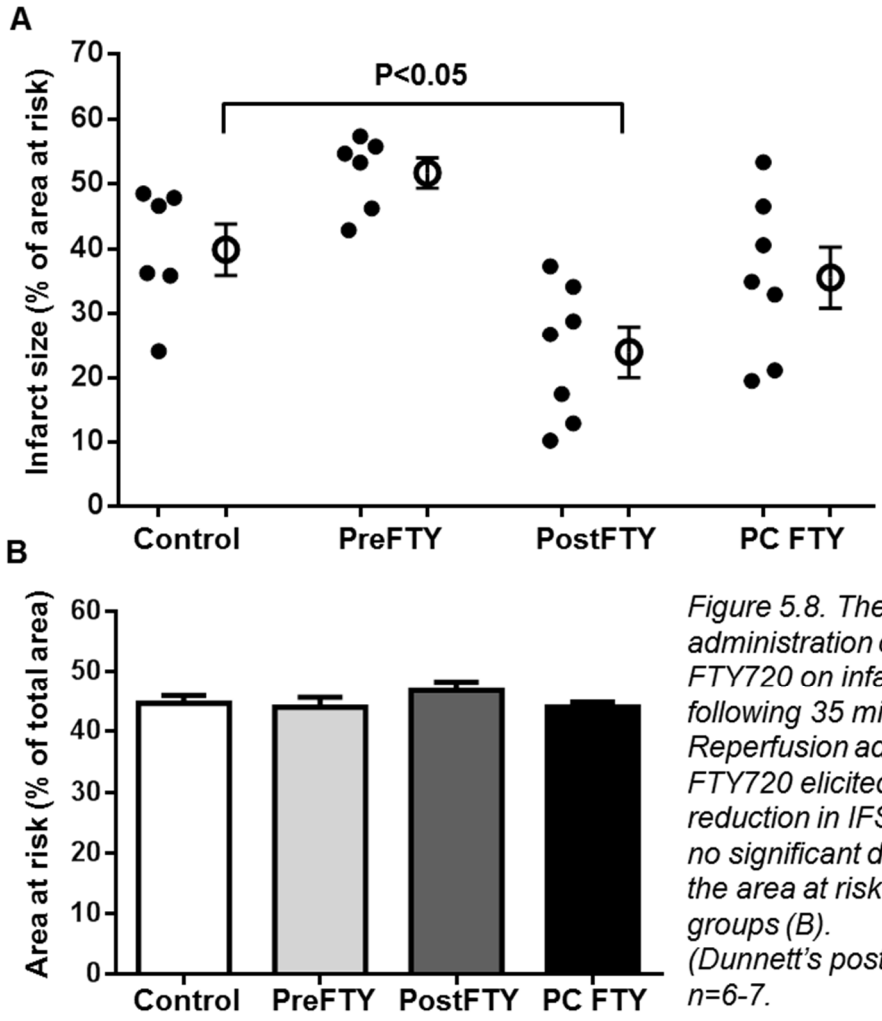


Figure 5.8. The effect of the administration of 1 μM FTY720 on infarct size following 35 minutes RI. (A) Reperfusion administration of FTY720 elicited a significant reduction in IFS. There were no significant differences in the area at risk between groups (B). (Dunnett's post hoc test). n=6-7.

Functional recovery of these hearts (table 5.3) deviated from the IFS data, since none of the interventions elicited any significant change in functional recovery, except for a lowering in CO associated with FTY720 preconditioning (Control: 46.56±10.25% vs. PC FTY: 21.55±4.38, n=6-7; P<0.05).

Table 5.3. Percentage functional recovery following the administration of 1 μM FTY720 to isolated rat heart preparations.
** P<0.05 vs. Control using Dunnett's post test.*

Group	Recovery as a percentage of baseline		n-value
	Aortic output	Cardiac output	
Control	27.86±13.22	46.56±10.25	6
PreFTY	17.04±6.07	28.29±6.22	6
PostFTY	6.72±3.77	26.59±6.84	7
PC FTY	6.58±3.13	21.55±4.38*	7

CO is a composite end-point which is determined by multiple aspects of function. We therefore also analysed the degree of recovery of several other functional parameters (table 5.4), in order to

ascertain why CO was reduced in the preconditioning group. Although all functional parameters measured tended to be lower in the PC FTY group, nothing was statistically reduced except the degree of heart rate (HR) recovery (percentage HR recovery: Control: 95.00±1.85% vs. PC FTY: 59.39±12.35%, n=6-7; T-test: P<0.05). It therefore seems that preconditioning with FTY720 suppressed the recovery of contractile function, specifically HR, contributing to a reduction in CO.

Table 5.4. Analyses of different aspects involved in functional recovery of control and FTY720 preconditioned (1 µM) hearts.
† Coronary flow measured during work mode.
** NS: non-significant*

Group	Recovery as a percentage of baseline				n-value
	Coronary flow†	Total work	Heart rate	Systolic pressure	
Control	106.80±6.68	40.74±9.98	95.00±1.85	84.79±2.70	6
PC FTY	69.41±17.17	19.83±4.04	59.39±12.35	78.81±13.17	7
Significance	NS*	NS	P<0.05	NS	

In summary, 1 µM FTY720 exerted an infarct sparing effect when administered at reperfusion, but unexpectedly increased IFS when administered immediately prior to ischaemia. This effect was abrogated when 5 minutes normal perfusion was applied between FTY720 administration and sustained ischaemia (a preconditioning protocol). Preconditioning with FTY720 could however not reduce IFS compared to control, and in fact functional recovery in these hearts were compromised relative to control, especially with regards to CO and HR. Functional recovery in all other groups were comparable to control and did not mirror the observed changes in IFS.

The effect of 2.5 µM FTY720 on infarct size and associated functional recovery

The effects of 2.5 µM FTY720 administration on I/R injury in the isolated rat heart are shown in Figure 5.9. Reperfusion administration of 2.5 µM FTY720 exerted a relatively small, yet significant, infarct sparing effect similar to what was observed with FTY720 at a concentration of 1 µM FTY720 (Control: 39.89±3.93% vs. PostFTY: 30.45±2.1.6, n=6; P<0.05). In contrast to 1 µM FTY720, pre-ischaemic treatment with 2.5 µM FTY720 was however associated with a significant reduction in IFS (Control: 39.89±3.93% vs. PostFTY: 29.97±1.03%, n=6; P<0.05). It is therefore clear that FTY720 elicits a dose-dependent response. Since pre-ischaemic FTY720 administration already exerted a cardioprotective effect, we did not include a preconditioning protocol for 2.5 µM FTY720.

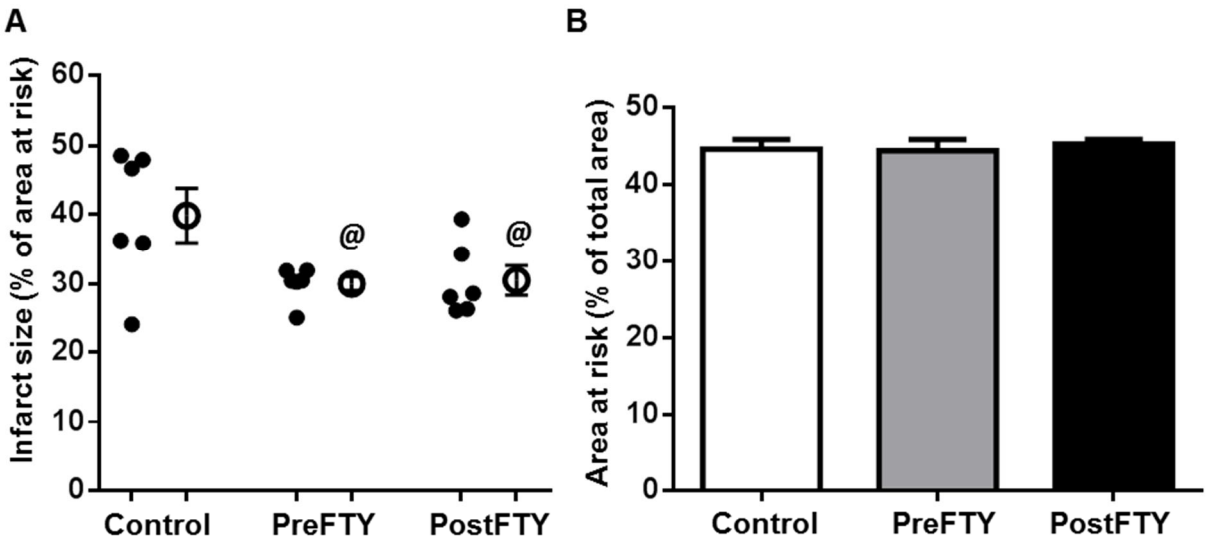


Figure 5.9. The effect of the administration of 2.5 µM FTY720 on infarct size following 35 minutes RI. Both pre-ischaemic, as well as reperfusion treatment elicited a significant reduction in IFS (A). There were no significant differences in the area at risk between groups (B).
Dunnett's post hoc test; @ P<0.05 vs. control; n=6.

Despite 2.5 µM FTY720 exerting an infarct sparing effect, both as a pre-ischaemic treatment as well as during reperfusion, functional recovery in these hearts was severely compromised as shown in table 5.5. Both pre-ischaemic and reperfusion administration of 2.5 µM FTY720 was associated with a profound reduction in functional recovery, as is evident by the fact that of the four hearts treated with FTY720 prior to ischaemia only one was able to produce an aortic output during reperfusion. Similarly, in the group treated with FTY720 at the onset of reperfusion, only one of the six hearts produced a measurable aortic output. Further analysis of the different components contributing to AO and CO reveals that although all parameters appeared suppressed (table 5.6) it was only coronary flow and total work which were significantly reduced in the reperfusion administration group (Coronary flow: Control: 106.80±6.68% vs. PostFTY: 53.96±18.27, n=6; P<0.05, and Total work: 40.74±9.98% vs. PostFTY: 11.84±4.59%).

Table 5.5. Percentage functional recovery following the administration of 2.5 µM FTY720 to isolated rat heart preparations.
** P<0.05 vs. Control by using Dunnett's correction.*

Group	Recovery as a percentage of baseline		n-value
	Aortic output	Cardiac output	
Control	27.86±13.22	46.56±10.25	6
PreFTY	0.93±0.93	30.73±3.83	4
PostFTY	2.08±2.08	15.02±5.38*	6

Table 5.6. The effects of 2.5 µM FTY720 administration on different aspects of recovery following 35 minutes regional ischaemia.

† Coronary flow measured during work mode.
**P<0.05 vs. Control (Dunnett's test)*

Group	Recovery as a percentage of baseline				n-value
	Coronary flow†	Total work	Heart rate	Systolic pressure	
Control	106.80±6.68	40.74±9.98	95.00±1.85	84.79±2.69	6
PreFTY	112.00±9.34	22.60±4.93	85.37±7.97	73.34±11.30	4
PostFTY	53.96±18.27*	11.84±4.59*	60.73±19.46	50.96±16.58	6

In summary, FTY720 at 2.5 µM exerted a paradoxical effect: on the one hand both pre-ischaemic and reperfusion treatment reduced IFS, while on the other hand, both these administration protocols significantly reduced functional ability during reperfusion. In the case of reperfusion administration, these reductions in functional recovery were mediated by a reduction in coronary flow and total work. This indicates that FTY720 has a protective effect with regards to cell death, but detrimental effects regarding functional recovery.

The effect of PP2A activation by the administration of FTY720 on functional recovery

Having established that both 1 µM and 2.5 µM FTY720 administered directly before sustained ischaemia or at the onset of reperfusion exerted dose-specific effects on IFS we decided to investigate these administration protocols in the context of a perfusion approach specifically aimed at assessing functional recovery after 20 minutes of GI. This model has the additional advantage that it generates a homogeneous heart preparation which is ideally suited for protein determination and profiling experiments using Western blotting techniques.

For these experiments we followed the same approach as for the IFS determination experiments: FTY720 was administered either directly before sustained ischaemia, or at the onset of reperfusion (Figure 5.5).

The effect of 1 µM FTY720 on functional recovery following 20 minutes global ischaemia

The baseline pre-ischaemic data for these hearts are shown in table 5.7. There were no significant differences between any of the FTY720 administration groups relative to control.

Table 5.7. Pre-ischaemic functional parameters of hearts before exposure to 20 minutes of GI (FTY720 1 µM).
@ Coronary flow measured during pre-ischaemic retrograde perfusion.

Group	Coronary flow (ml/min)@	Aortic output (ml/min)	Cardiac output (ml/min)	Total work (mW)	n-value
Control	8.45±0.75	44.20±3.41	60.55±4.10	13.17±1.22	5-10
PreFTY	9.13±1.13	47.13±4.11	63.81±4.78	14.41±0.56	5-8
PostFTY	6.70±0.47	51.50±1.78	66.39±2.45	14.02±0.74	9-10

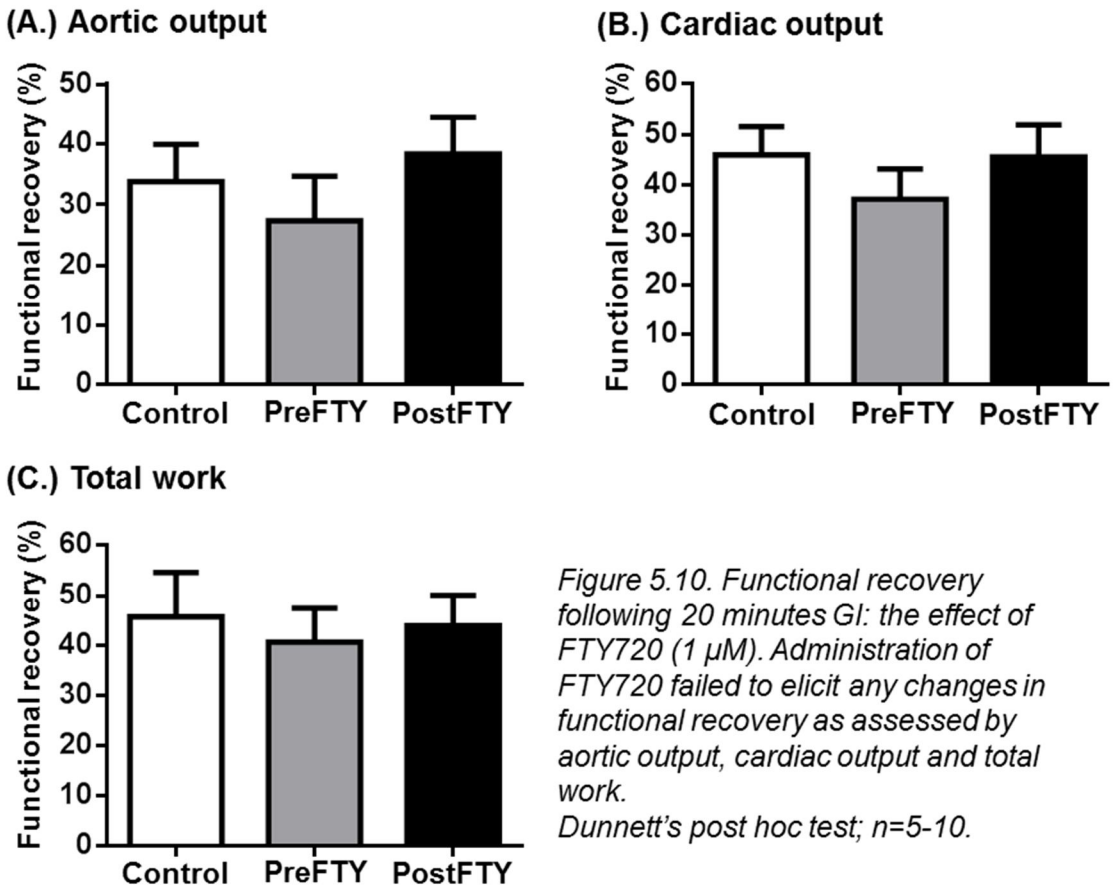


Figure 5.10. Functional recovery following 20 minutes GI: the effect of FTY720 (1 µM). Administration of FTY720 failed to elicit any changes in functional recovery as assessed by aortic output, cardiac output and total work.
Dunnett's post hoc test; n=5-10.

Functional recoveries following 20 minutes GI are shown in Figure 5.10. In agreement with functional recovery data from the IFS study, FTY720 administration at 1 µM failed to elicit any effects on functional recovery relative to control. In contrast to RI, under these conditions of GI, FTY720 had no effect on HR (Percentage heart rate recovery: Control: 85.00±6.12% vs. PreFTY: 93.21±6.19% and PostFTY: 69.05±13.44%, n=8-9; non-significant).

The effect of 2.5 µM FTY720 on functional recovery following 20 minutes global ischaemia

Baseline values for these experiments are shown in table 5.8. There were no significant differences between control and any of the experimental FTY720 groups, except for coronary flow which was lower in the PostFTY group compared to control (Control: 8.45±0.75 ml/min vs. PostFTY: 5.50±0.49 ml/min, n=7-10; P<0.01). All the other functional parameters in the PostFTY group were however comparable to control. It is therefore unlikely that the lower CF value could be a confounding factor in terms of the experimental outcomes.

Table 5.8. Pre-ischaemic functional ability of isolated rat hearts before exposure to 20 minutes GI (FTY720; 2.5 µM).
@ Coronary flow measured during pre-ischaemic retrograde perfusion.
 # P<0.01 vs. control.

Group	Coronary flow (ml/min)[@]	Aortic output (ml/min)	Cardiac output (ml/min)	Total work (mW)	n-value
Control	8.45±0.75	44.20±3.41	60.55±4.10	13.17±1.22	5-10
PreFTY	6.50±0.52	41.00±5.21	55.25±6.48	13.98±0.41	4-6
PostFTY	5.50±0.49[#]	44.29±2.88	58.93±4.81	11.54±1.09	6-7

The effect of 2.5 µM FTY720 on functional recovery during reperfusion is shown in Figure 5.11. Pretreatment with the drug at 2.5 µM was associated with a profound reduction in aortic output (Control: 33.88±6.12% vs. PreFTY: 0%, n=6-10; P<0.001), cardiac output (Control: 45.94±5.57% vs. PreFTY: 12.20±2.68%, n=6-10; P<0.001) and total work (Control: 45.67±8.98% vs. PreFTY: 2.79±2.79%, n=4-5; P<0.01).

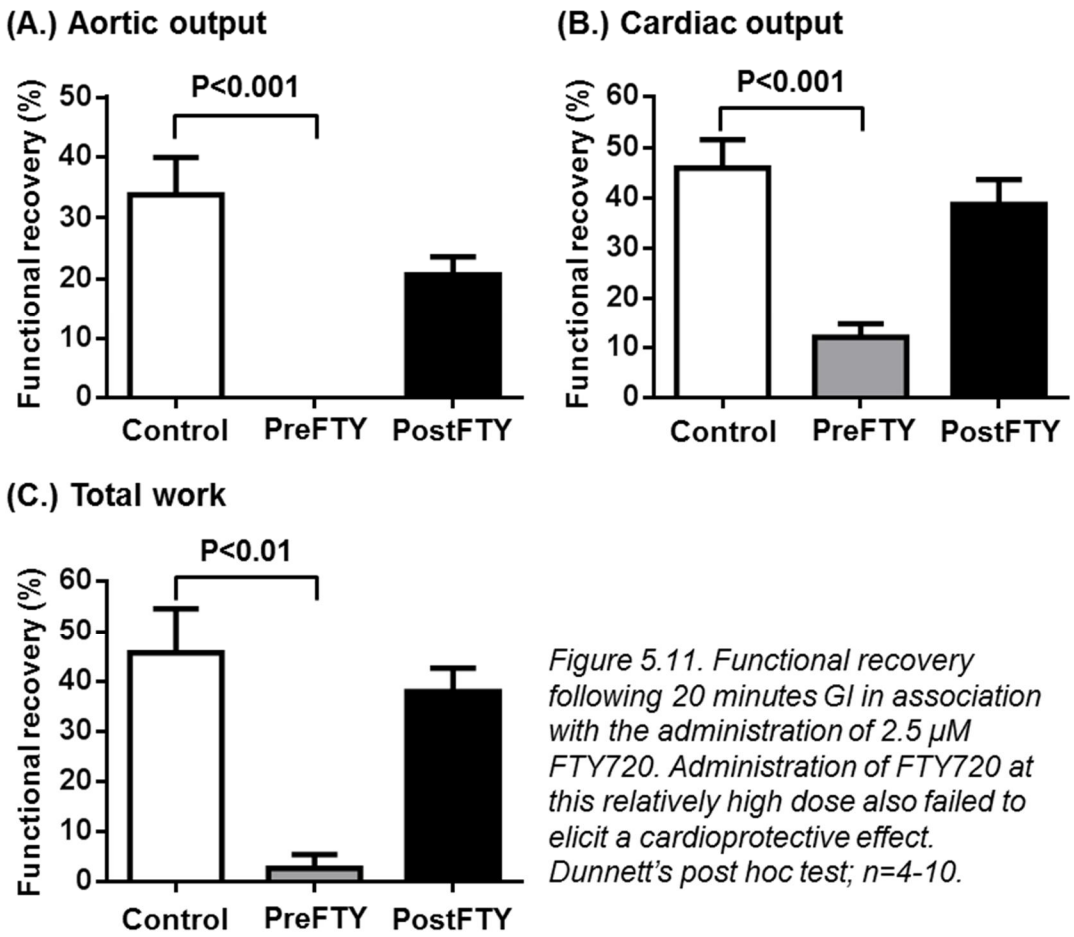


Figure 5.11. Functional recovery following 20 minutes GI in association with the administration of 2.5 µM FTY720. Administration of FTY720 at this relatively high dose also failed to elicit a cardioprotective effect. Dunnett's post hoc test; n=4-10.

The detrimental effects of pretreatment with FTY720 are further analysed in table 5.9.

Table 5.9. The effects of 2.5 µM FTY720 administration on different aspects of recovery following 20 minutes of GI.
† Coronary flow measured during work mode.
@ P<0.0001 vs. Control (Dunnett's test)
 # P<0.05 vs. Control (Dunnett's test)

Group	Recovery as a percentage of baseline			n-value
	Coronary flow†	Heart rate	Systolic pressure	
Control	72.27±8.97	85.00±6.11	88.13±4.45	8-10
PreFTY	45.19±7.25#	10.53±10.53@	15.10±15.10@	6
PostFTY	104.00±5.80	92.94±3.77	87.99±3.64	7

Pre-ischaemic treatment with 2.5 µM FTY720 suppressed all functional parameters during reperfusion. In fact, of the group of six hearts pretreated with FTY720, only one recovered sufficiently to record heart rate and pressure data, while the other five hearts presented with zero percent recovery.

The effects of FTY720 on coronary flow

During our initial experiments to determine the effects of FTY720 on PP2A activity, as well as during the IFS study, we noticed that FTY720 exerted a potent, but transient effect on CF by dramatically increasing it. We therefore utilized the 20 minutes GI (functional recovery alone) study to monitor the effects of FTY720 on CF at different time points during the perfusion protocol and expressing it relative to pre-ischaemic stabilisation values. All CF values were measured during retrograde perfusion episodes in the perfusion protocol.

Pretreatment with FTY720

Pre-ischaemic treatment with both 1 µM and 2.5 µM FTY720 elicited a potent increase in CF (Figure 5.12 A) as measured at the end of the FTY720 administration period (Control: 0.95±0.05 AU vs. PreFTY1: 2.25±0.27 AU and PreFTY2.5: 2.56±0.27 AU, n=5-8; $P<0.01$). Unexpectedly pretreatment also led to a reduction in the CF over the first 15 minutes of reperfusion (Figure 5.12 B: Control: 1.94±0.17 AU vs. PreFTY1: 1.60±0.08 AU and PreFTY2.5: 1.08±0.05 AU, n=3-5; $P<0.05$) in a dose dependent manner, since 2.5 µM FTY720 elicited a greater reduction in CF than 1 µM (PreFTY1: 1.60±0.08 AU vs. PreFTY2.5: 1.08±0.05 AU, n=3-5; T-test: $P<0.01$). This effect was however shortlived and at the end of retrograde reperfusion (20 minutes reperfusion) there were no differences between any of the groups (Figure 5.12 C). Pretreatment hearts therefore had an increased CF relative to control at the onset of ischaemia, but lower CF at the onset of reperfusion.

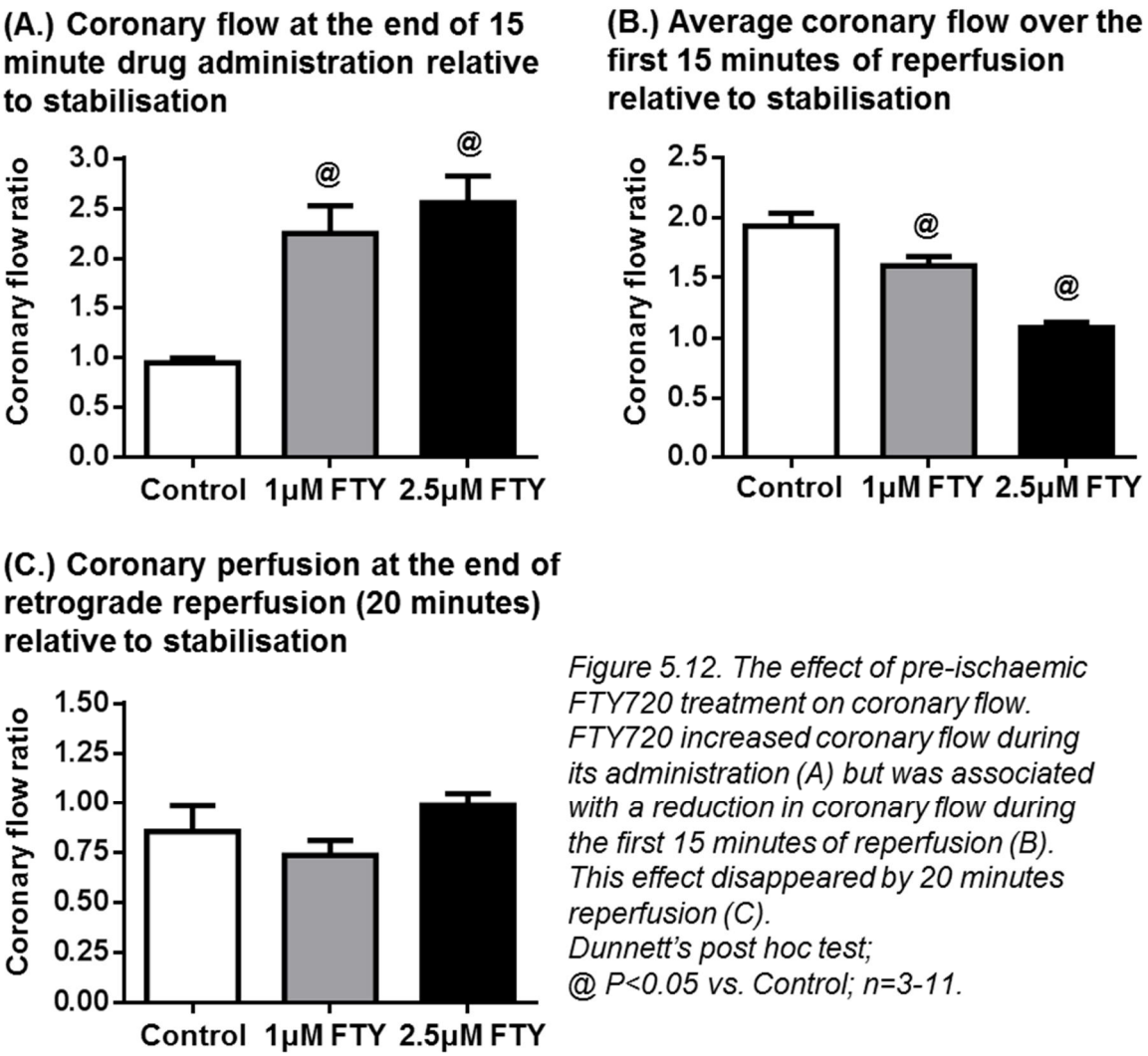


Figure 5.12. The effect of pre-ischaemic FTY720 treatment on coronary flow. FTY720 increased coronary flow during its administration (A) but was associated with a reduction in coronary flow during the first 15 minutes of reperfusion (B). This effect disappeared by 20 minutes reperfusion (C). Dunnett's post hoc test; @ P<0.05 vs. Control; n=3-11.

Reperfusion treatment with FTY720

As expected, FTY720 administration during reperfusion (Figure 5.13) was associated with a significant increase in CF (Control: 1.21±0.11 AU vs. PostFTY1: 2.59±0.18 AU and PostFTY2.5: 2.19±0.15 AU, n=4-9; P<0.01) which still persisted 5 minutes later at the end of retrograde reperfusion (Control: 0.85±0.13 AU vs. PostFTY1: 1.86±0.15 AU and PostFTY2.5: 1.77±0.17 AU, n=7-11; P<0.001).

The effects of FTY720 on CF is summarised in Figure 5.13 C. Administration of FTY720 (1 and 2.5 µM) consistently increased coronary flow during drug administration. This effect was however transient and in the pretreatment groups (1 and 2.5 µM) there was in fact a reduction in coronary flow at initial reperfusion (the first 15 minutes of reperfusion), which stabilized at control values by the end of retrograde perfusion (20 minutes). Our data therefore indicate that FTY720, at both doses studied, exerted a potent direct vasodilatory effect in the isolated rat heart. Intriguingly, this vasodilatory effect was replaced by vasoconstriction with the addition of I/R to FTY720, as seen in the pre-ischaemic treatment groups.

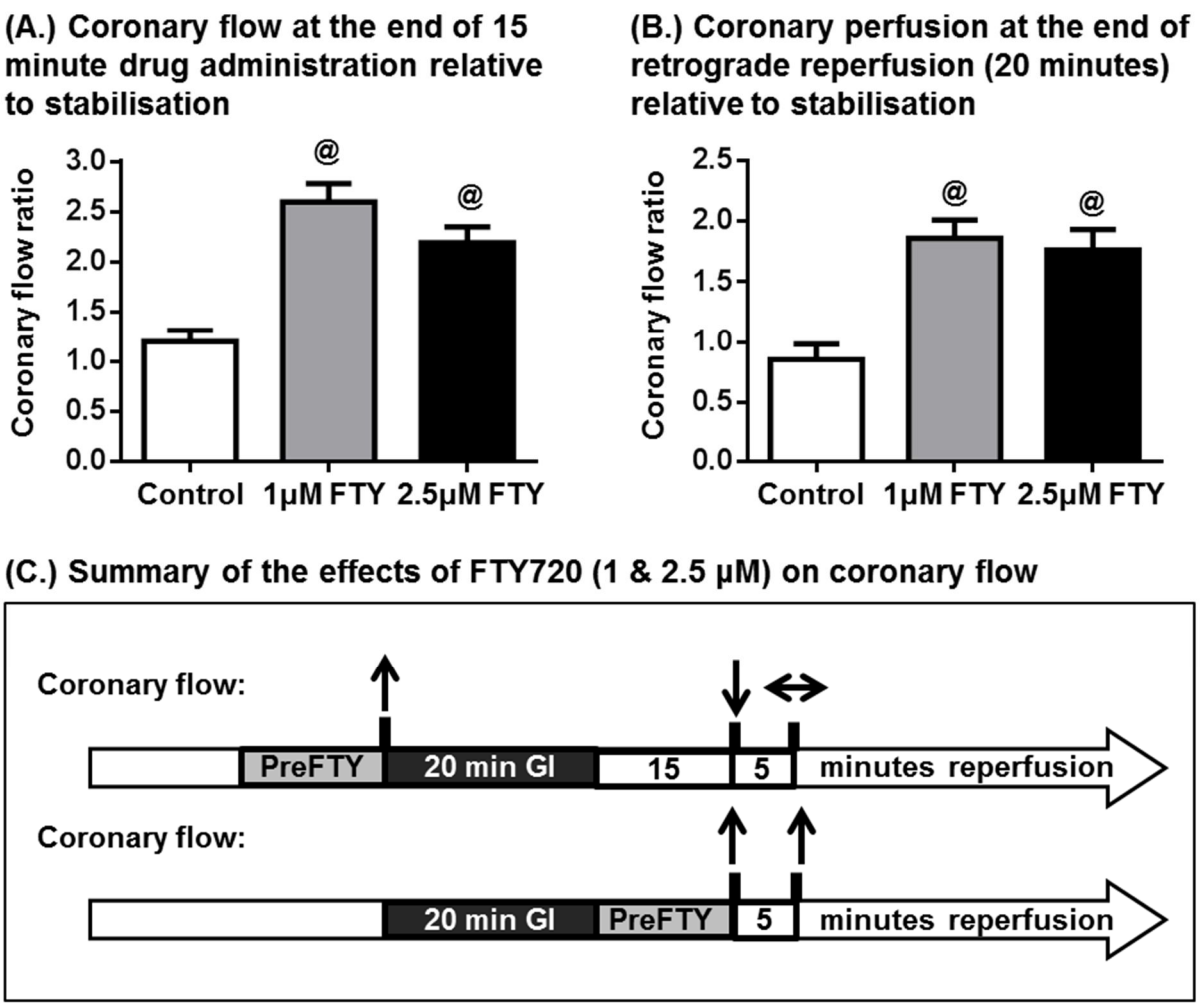


Figure 5.13. (A&B) The effect of FTY720 given at the onset of reperfusion on coronary flow. FTY720 signifcantly increased coronary flow in comparison to control (A). This effect was still evident at the end of retrograde reperfusion (B). Dunnett's post hoc test; @ P<0.01 vs. control; n=4-11.
(C) Both 1 and 2.5 µM FTY720 exerted the same pattern of effects on coronary flow. Essentially, coronary flow increased during FTY720 administration, but in the pretreatment group was reduced in the first minutes of reperfusion following global ischaemia whereafter it normalized.

Protein profiles associated with pre-ischaemic FTY720 treatment

Our initial interest in the effects of FTY720 on the heart exposed to I/R had two components to it: Firstly, not much work has been done regarding FTY720 in this setting *per se*; while secondly, FTY720 administration has been linked to an increase in the activity of PP2A. Our initial studies concerning the effects of FTY720 on IFS and functional recovery addressed both these aspects, since we employed two different concentrations of FTY720, which revealed dose-depency with regards to the effects of FTY720.

Concerning the effects of FTY720 on signalling protein levels and phosphorylation, the aim was to specifically focus on the contribution of PP2A activation. For these experiments we therefore

investigated a single dose of FTY720, namely 1 µM: a relatively low dose yet still capable of activating PP2A (see Figure 5.7).

Pre-treatment with FTY720: Protein profiles at the onset of ischaemia

Hearts were perfused for 15 minutes with 1 µM FTY720 and immediately freeze-clamped before the onset of sustained ischaemia.

Protein phosphatase 2A

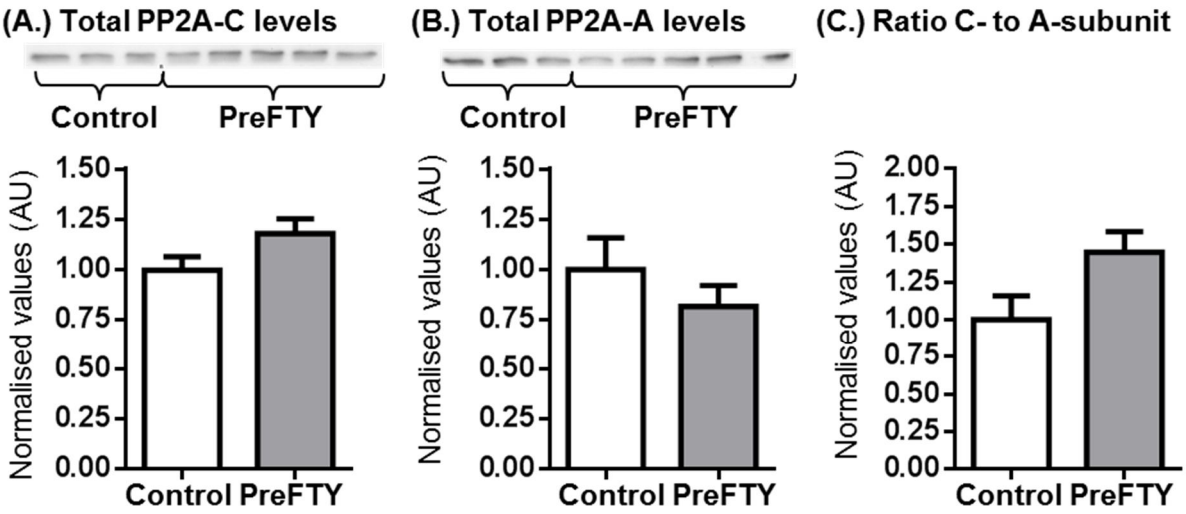


Figure 5.14. Expression of PP2A-A and PP2A-C at the end of a 15 minute period of FTY720 administration.
FTY720 on its own, in the absence of ischaemia or reperfusion, failed to elicit an effect on PP2A levels. n=3-5.

Similar to initial results concerning the effect of FTY720 administration on PP2A under base-line conditions (Figure 5.7) we found that 15 minutes of exposure to FTY720 did not elicit any changes in the levels of PP2A-C, PP2A-A or the ratio between the two (Figure 5.14). FTY720 however reduced the level of phosphorylation of PP2A (Figure 5.15: Absolute phosphorylation: Control: 1.00±0.05 AU vs. PreFTY: 0.86±0.18 AU, n=3-5, $P<0.05$; Phosphorylated relative to total PP2A-C: Control: 1.00±0.02 AU vs. PreFTY: 0.74±0.06 AU, n=3-5, $P<0.05$), indicative of an increase in the activity of PP2A, thereby confirming the efficacy of this dose FTY720. Interestingly, despite exerting an effect on the phosphorylation of PP2A-C, FTY720 failed to induce any changes in the degree of methylation of PP2A-C.

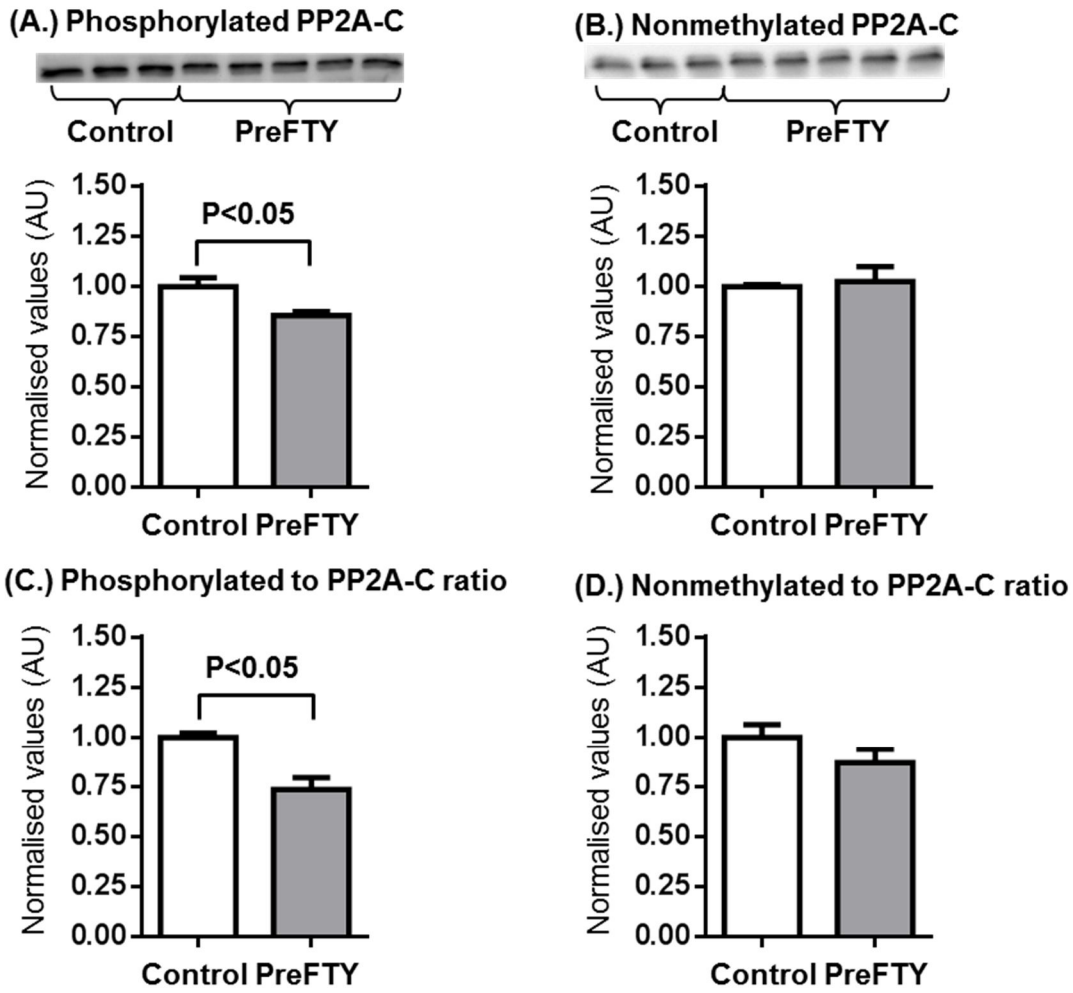


Figure 5.15. Phosphorylation and methylation of PP2A-C following 15 minutes exposure to 1 μM FTY720. FTY720 induced a reduction in the phosphorylation of PP2A-C (A&C), while not influencing the methylation of the enzyme. n=3-5.

Protein kinase B (Akt)

Despite activation of PP2A by FTY720, it was also associated with a potent increase in the phosphorylation, and therefore activity of PKB/Akt (Figure 5.16), both in absolute terms (Control: 1.00±0.06 AU vs. PreFTY: 1.61±0.09 AU, n=3-5, P<0.01) as well as in relation to the total levels of PKB/Akt (Control: 1.00±0.06 AU vs. PreFTY: 1.99±0.18 AU, n=3-5, P<0.01).

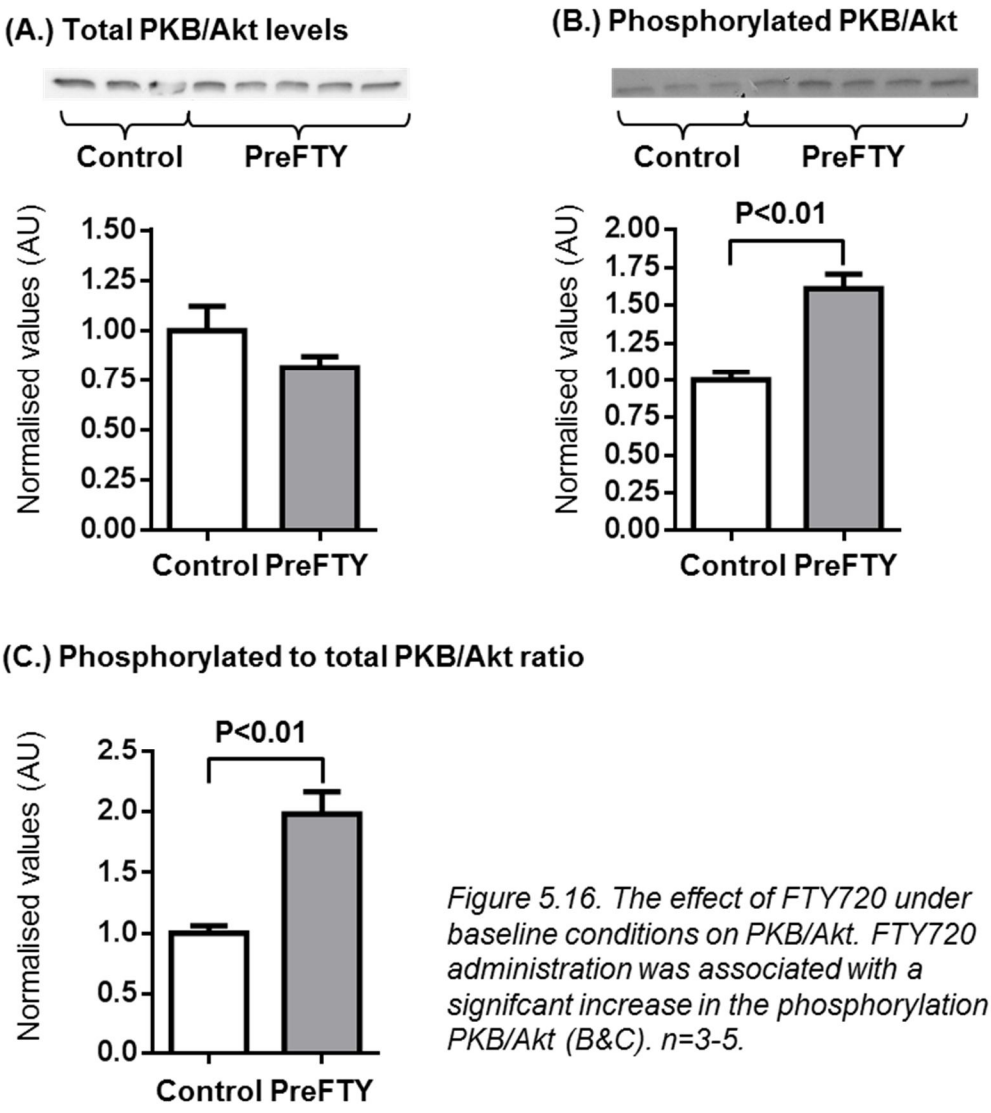


Figure 5.16. The effect of FTY720 under baseline conditions on PKB/Akt. FTY720 administration was associated with a signifcant increase in the phosphorylation of PKB/Akt (B&C). n=3-5.

Glycogen synthase kinase-3β

Although FTY720 exerted a strong stimulatory effect on PKB/Akt phosphorylation, this was not associated with a similar increase in the phosphorylation of GSK-3β (Figure 5.17). FTY720 failed to exert any significant effect on GSK-3β.

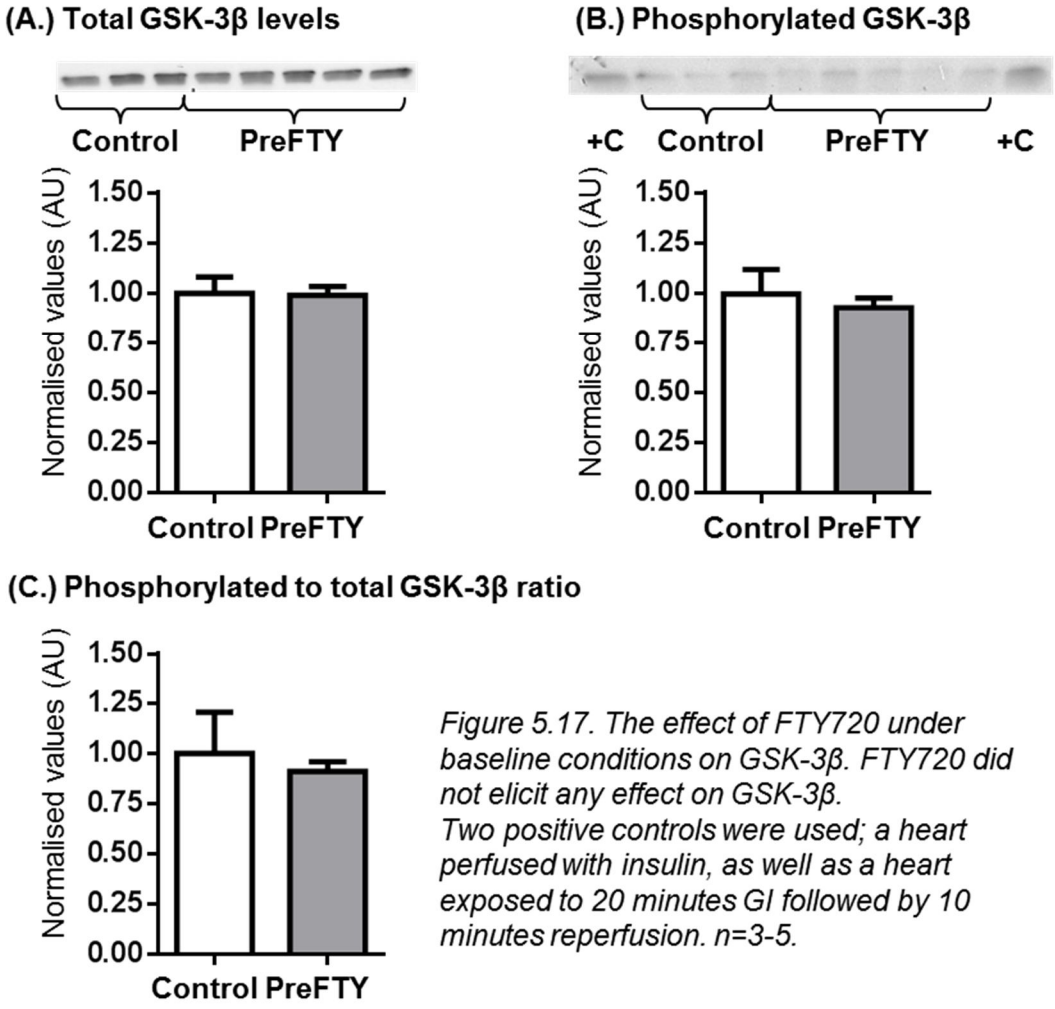


Figure 5.17. The effect of FTY720 under baseline conditions on GSK-3β. FTY720 did not elicit any effect on GSK-3β. Two positive controls were used; a heart perfused with insulin, as well as a heart exposed to 20 minutes GI followed by 10 minutes reperfusion. n=3-5.

p38 Mitogen activated protein kinase

Not only did FTY720 activate both PP2A, as well as PKB/Akt, but it was also associated with a very potent increase in the phosphorylation of p38 MAPK (Figure 5.18), again with regards to both absolute phosphorylation (Control: 1.00±0.08 AU vs. PreFTY: 2.77±0.52 AU, n=3-4, P<0.05), as well as the degree of phosphorylation relative to the total levels of p38 MAPK (Control: 1.00±0.07 AU vs. PreFTY: 2.89±0.53 AU, n=3-4, P<0.05).

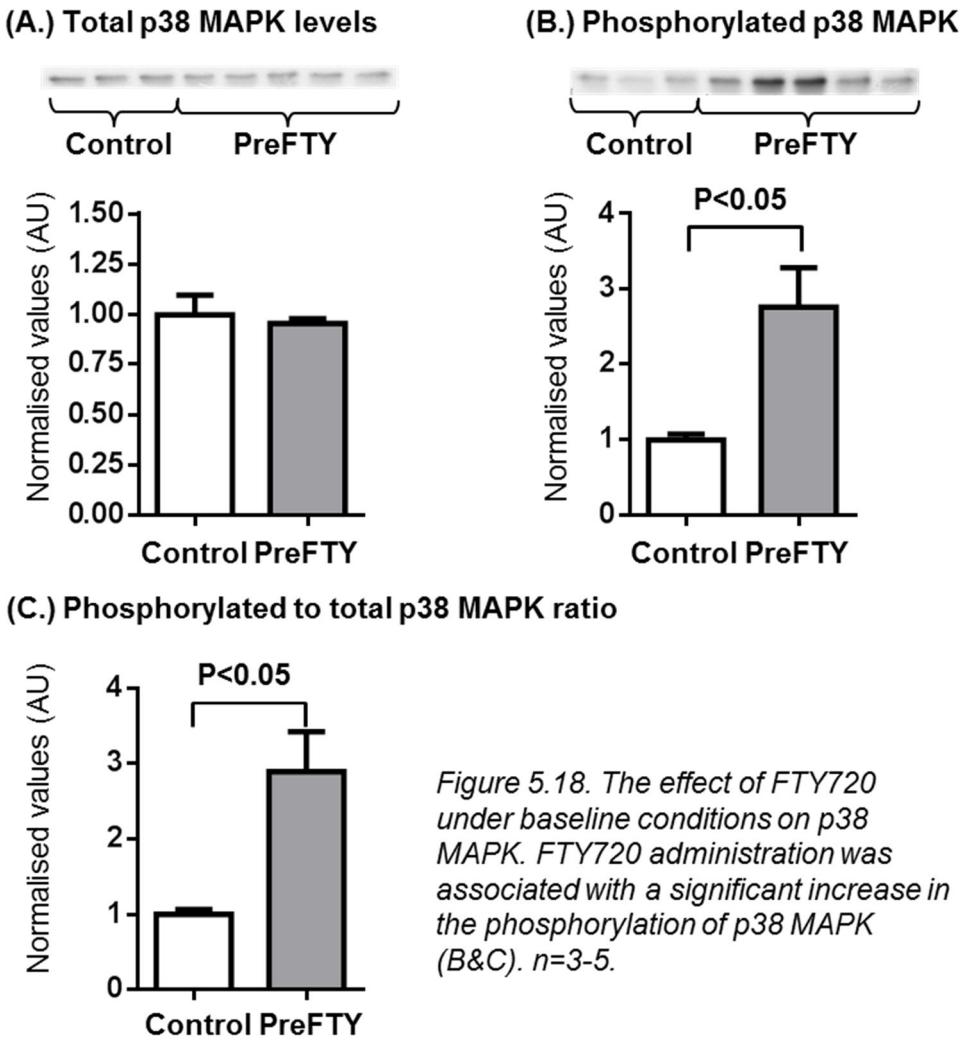


Figure 5.18. The effect of FTY720 under baseline conditions on p38 MAPK. FTY720 administration was associated with a significant increase in the phosphorylation of p38 MAPK (B&C). n=3-5.

Extracellular signal-regulated kinase p42/p44

In combination with the activation of PKB/Akt, FTY720 also induced an increase in the phosphorylation of the other RISK pathway signalling component, namely ERK p42/p44 (Figure 5.19). FTY720 elicited a potent increase in the phosphorylation of both ERK p42 (Absolute phosphorylation: Control: 1.00±0.14 AU vs. PreFTY: 2.52±0.24 AU, n=3-5, P<0.01; Phosphorylated relative to total ERK p42: Control: 1.00±0.18 AU vs. PreFTY: 3.17±0.43 AU, n=2-5, P<0.05), as well as ERK p44 (Absolute phosphorylation: Control: 1.00±0.29 AU vs. PreFTY: 2.53±0.26 AU, n=3-5, P<0.01; Phosphorylated relative to total ERK p44: Control: 1.00±0.02 AU vs. PreFTY: 2.07±0.31 AU, n=3-5, P<0.05).

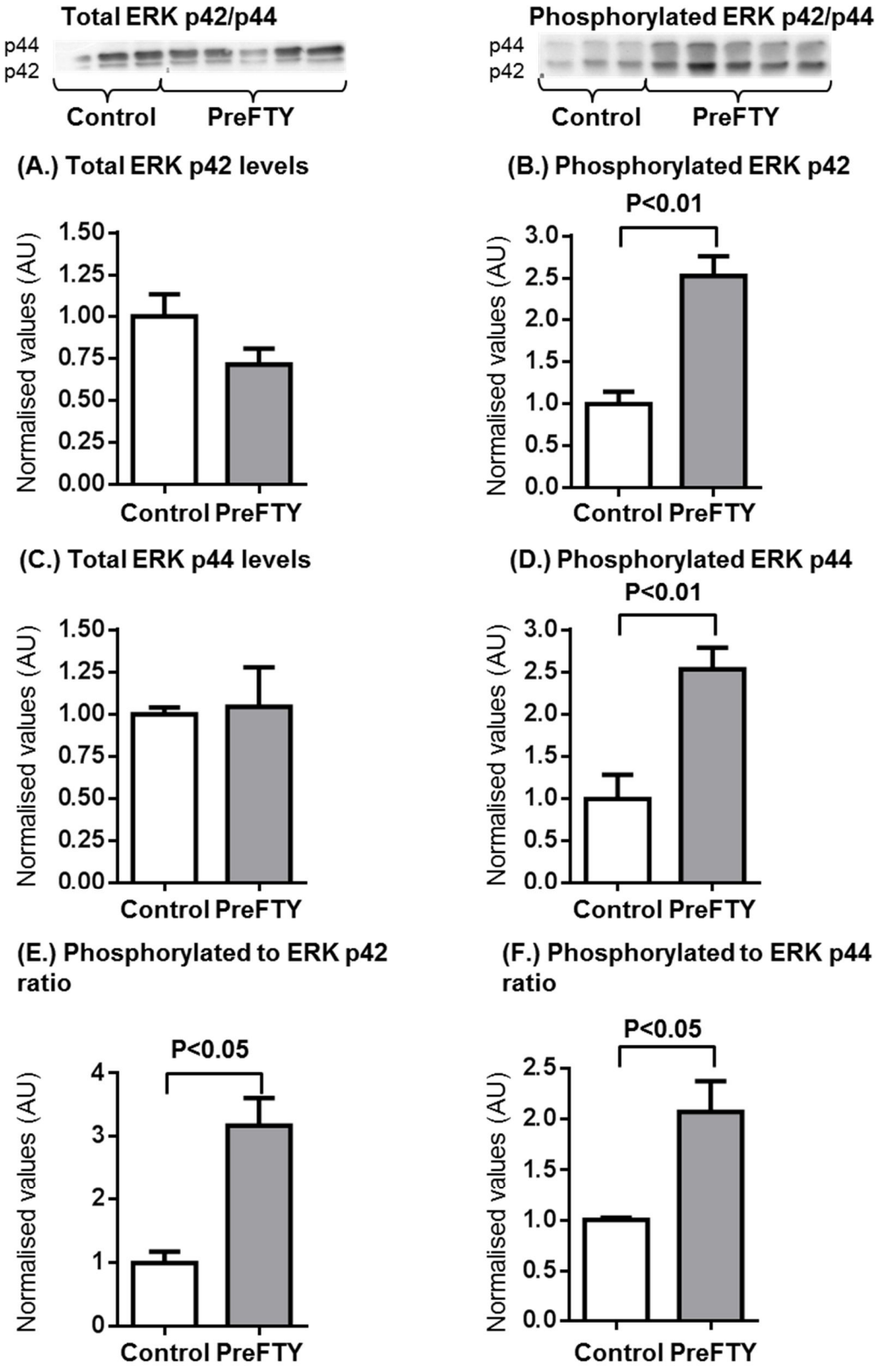


Figure 5.19. Effects of FTY720 treatment under baseline conditions on ERK p42/p44. FTY720 treatment per se *was associated with an increase in the phosphorylation of both ERK p42 (B&E), as well as ERK p44 (D&F). n=3-5.*

Equal loading: β-Tubulin

In order to confirm equal loading, a representative blot for the lysates was probed for β-Tubulin (Figure 5.20). No differences were detected between control and the FTY720 treatment groups.

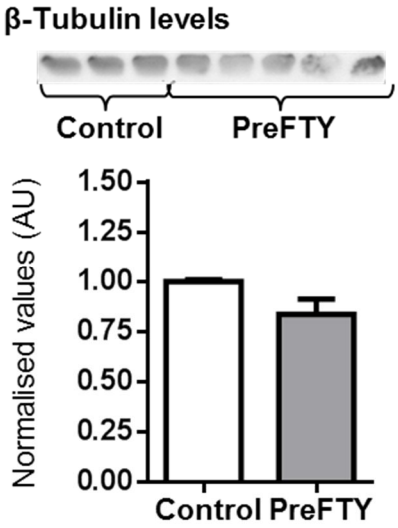


Figure 5.20. Loading control for the effects of FTY720 under pre-ischaemic conditions.
β-Tubulin was used as loading control for the determination of the kinase profiles associated with FTY720 treatment. n=3-5.

Pre-treatment with FTY720: Protein profiles at the end of 20 minutes GI

Following 15 minutes treatment with FTY720, hearts were exposed to 20 minutes GI and then snap frozen.

Protein phosphatase 2A

At the end of sustained ischaemia pretreatment with FTY720 was associated with an increase in the ratio of total PP2A-C to total PP2A-A (Control: 1.00±0.06 AU vs. PreFTY: 1.31±0.05 AU, n=3-5, P<0.01), although levels of neither of these proteins were significantly changed by FTY720 administration (Figure 5.21). In addition to this, FTY720 induced an increase in absolute phosphorylation relative to control (Control: 1.00±0.03 AU vs. PreFTY: 1.62±0.12 AU, n=3-6, P<0.05), although when expressed relative to total PP2A-C FTY720 exerted no additional effect relative to 20 minutes ischaemia (Control: 1.00±0.06 AU vs. 20 GI: 1.52±0.17 AU and PreFTY: 1.46±0.07 AU, n=3-5, P<0.05). Absolute nonmethylation was also increased in the FTY720 group relative to both control and 20 minutes ischaemia (Control: 1.00±0.05 AU and 20 GI: 1.15±0.04 AU vs. PreFTY: 1.53±0.09 AU, n=3-5; P<0.05), although when expressed relative to total PP2A-C it became evident that FTY720 did not elicit any effects additional to the effect already associated with 20 minutes GI (Control: 1.00±0.09 AU vs. 20 GI: 1.30±0.01 AU and PreFTY: 1.44±0.05 AU, n=3-5, P<0.05). It therefore seems that FTY720 induces the phosphorylation and demethylation of PP2A, although after 20 minutes GI this effect is no longer significant when expressed relative to the total pool of PP2A-C.

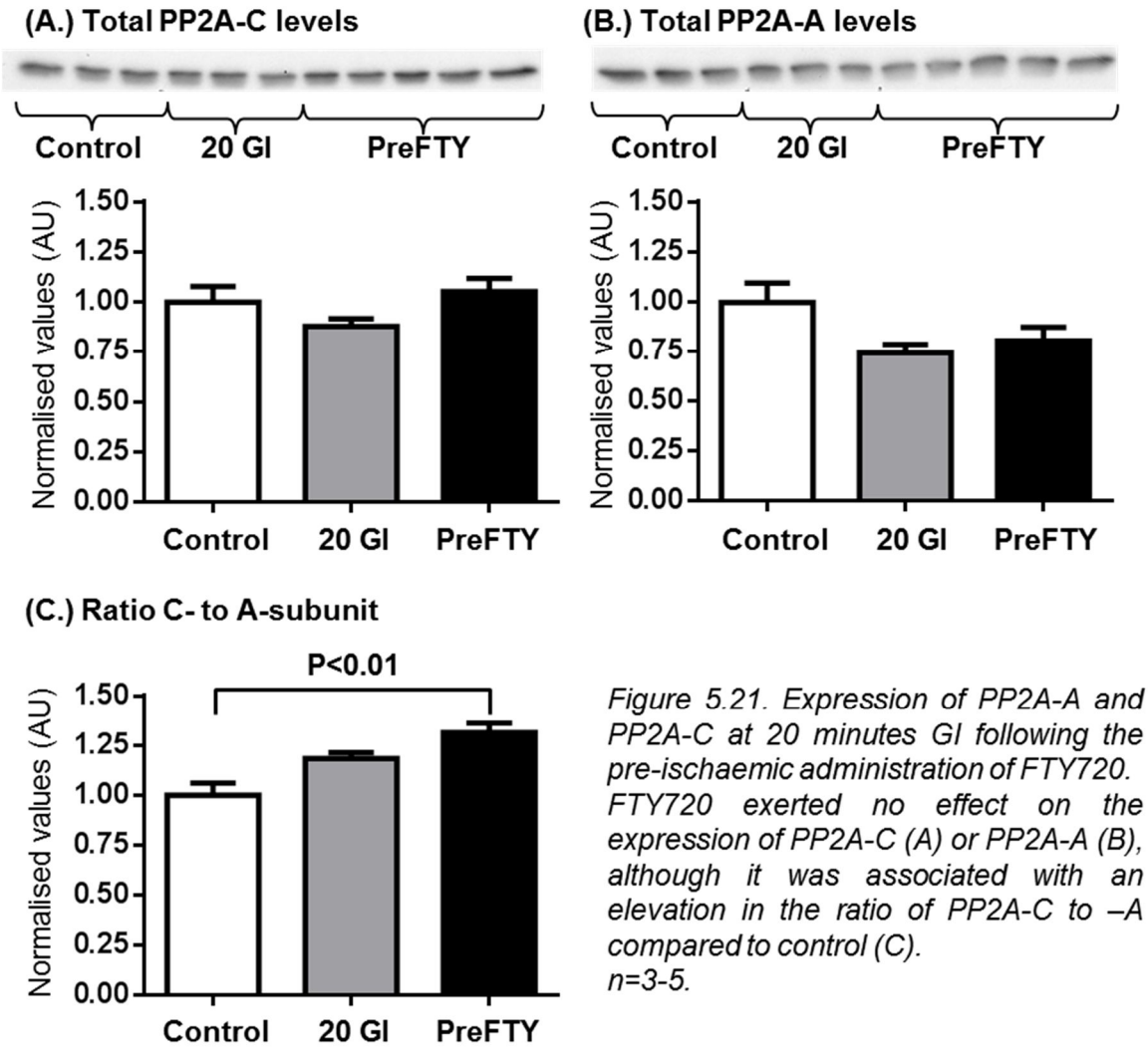


Figure 5.21. Expression of PP2A-A and PP2A-C at 20 minutes GI following the pre-ischaemic administration of FTY720. FTY720 exerted no effect on the expression of PP2A-C (A) or PP2A-A (B), although it was associated with an elevation in the ratio of PP2A-C to –A compared to control (C). n=3-5.

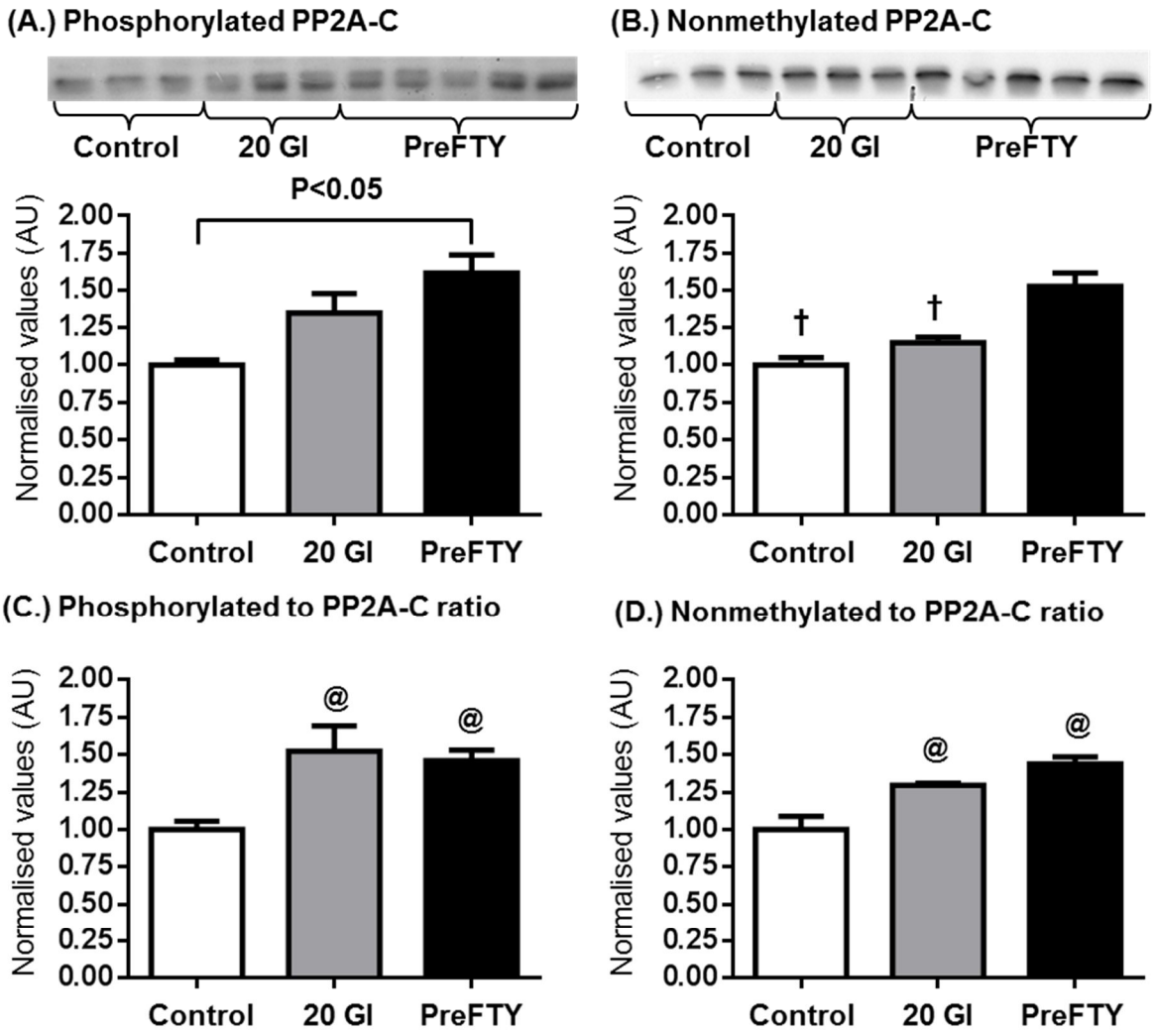


Figure 5.22. Phosphorylation and methylation of PP2A-C in hearts treated with FTY720 prior to 20 minutes GI.
Pretreatment with FTY720 increased the absolute levels of phosphorylation and nonmethylation of PP2A-C (A&B), although this effect was lost when expressed relative to total PP2A-C (C&D) . † P<0.05 vs. PreFTY; @ P<0.05 vs. Control; n=3-5.

Protein kinase B (Akt)

The pre-ischaemic administration of FTY720 was not associated with any changes in total PKB/Akt expression or phosphorylation at the end of 20 minutes GI (Figure 5.23).

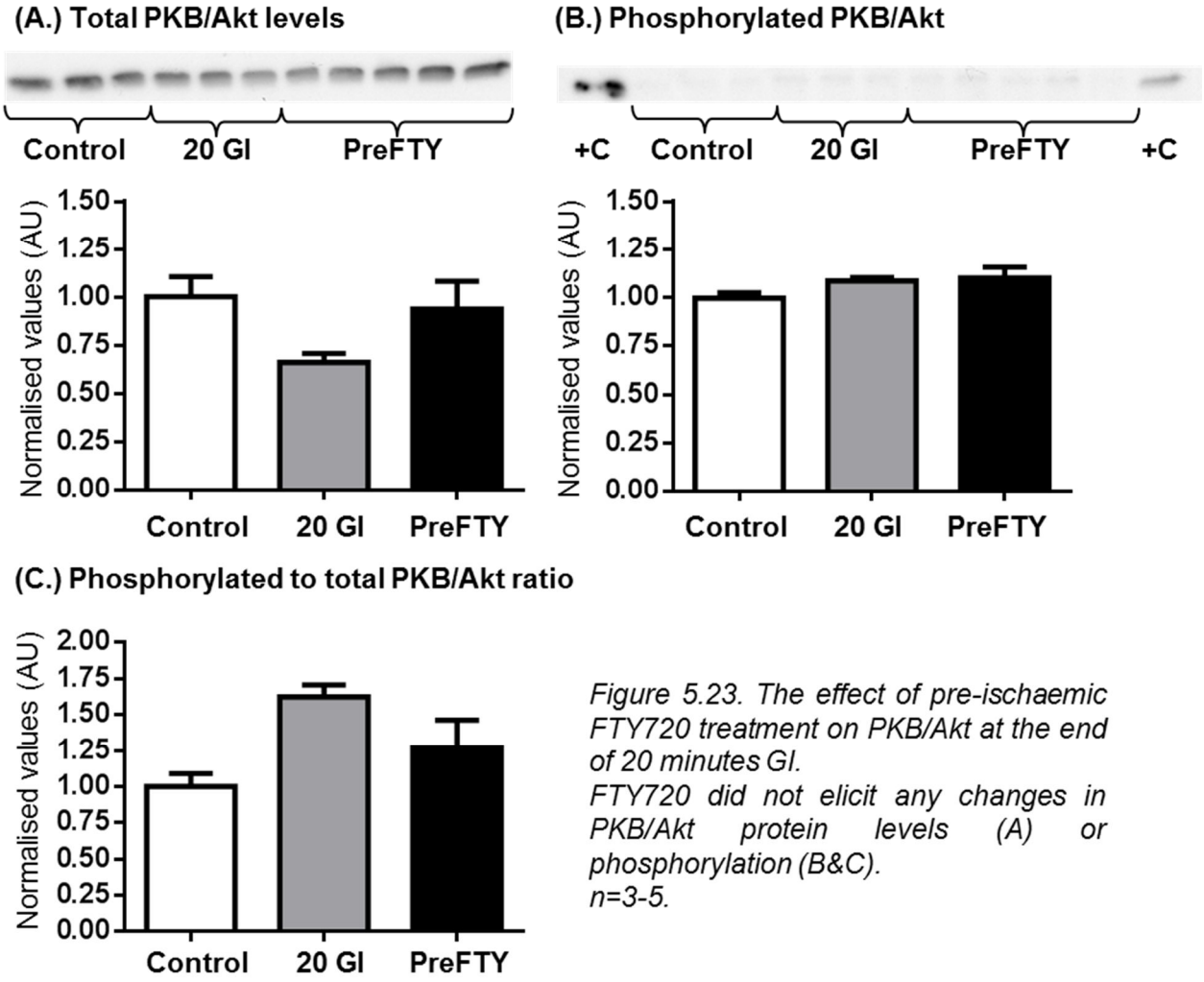


Figure 5.23. The effect of pre-ischaemic FTY720 treatment on PKB/Akt at the end of 20 minutes GI.
FTY720 did not elicit any changes in PKB/Akt protein levels (A) or phosphorylation (B&C).
n=3-5.

Glycogen synthase kinase-3β

Twenty minutes of GI activated GSK-3β (Phosphorylated GSK-3β relative to total GSK-3β: Control: 1.00±0.18 AU vs. 20 GI: 2.98±0.35, n=3; $P<0.05$), as was also noted in Chapter 4. Although PreFTY did not differ significantly from 20 minutes GI alone, it also did not differ significantly from control (Figure 5.24 B). In addition to this, an unpaired T-test directly comparing 20 minutes GI with PreFTY shows a bordeline significant difference between the two groups (20 GI: 2.98±0.35 AU vs. PreFTY720: 1.70±0.41 AU, n=3-4; $P=0.073$). Taken together our data suggests that FTY720 is associated with a reduction in the ischaemia-mediated phosphorylation of GSK-3β. It could be speculated that FTY720 elicits this effect through its function as an activator of PP2A.

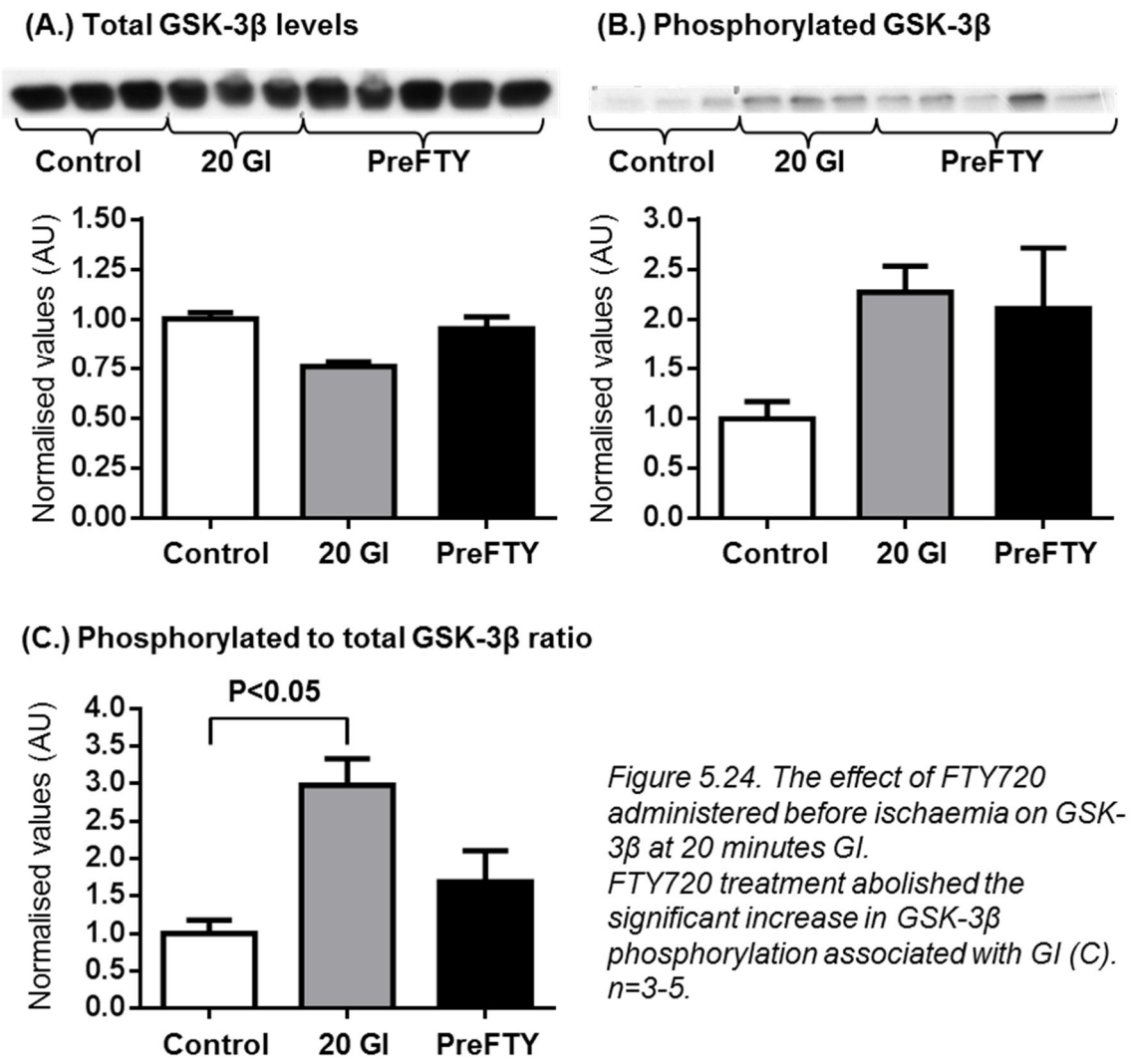


Figure 5.24. The effect of FTY720 administered before ischaemia on GSK-3β at 20 minutes GI. FTY720 treatment abolished the significant increase in GSK-3β phosphorylation associated with GI (C). n=3-5.

p38 Mitogen activated protein kinase

Pre-ischaemic treatment did not elicit any effect on the levels of total p38 MAPK (Figure 5.25). It also failed to elicit any effect regarding the ischaemia-associated increase in absolute phosphorylation levels of p38 MAPK (Control: 1.00±0.03 AU vs. 20 GI: 9.16±0.77 AU and PreFTY: 6.70±1.08 AU, n=3-5; P<0.01). However expression of phosphorylation levels relative to total p38 MAPK revealed that although both 20 minutes GI and PreFTY were still elevated above control levels (Control: 1.00±0.13 AU vs. 20 GI: 10.00±0.55 AU and PreFTY: 6.14±1.03 AU, n=3-5; P<0.05), PreFTY was associated with a lower relative degree of phosphorylation than ischaemia alone (20 GI: 10.00±0.55 AU vs. PreFTY: 6.14±1.03 AU, n=3-5; P<0.05). As was the case with GSK-3β, it therefore seems that FTY720 suppresses the ischaemia mediated phosphorylation of p38 MAPK, possibly through the activation of PP2A.

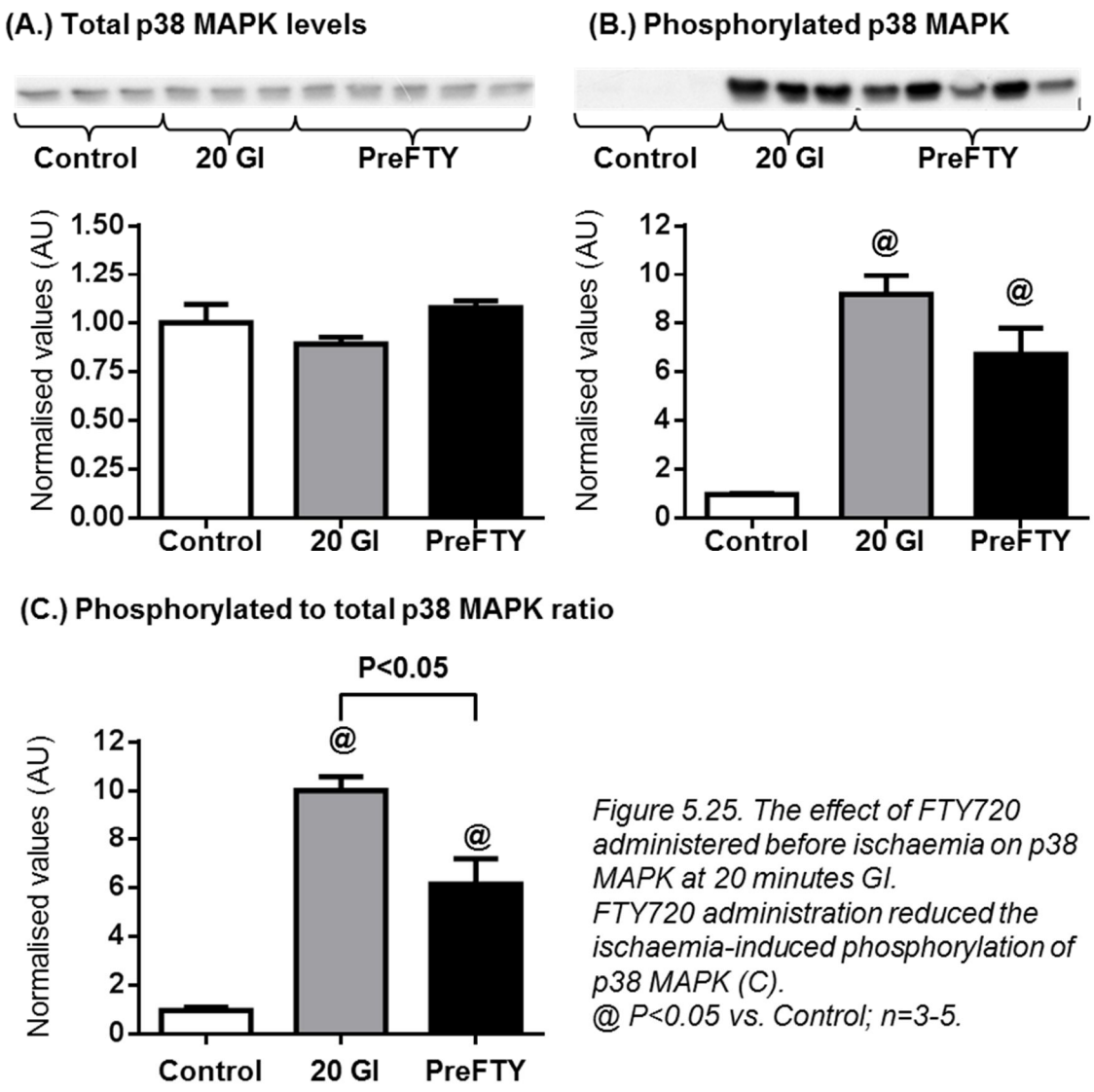


Figure 5.25. The effect of FTY720 administered before ischaemia on p38 MAPK at 20 minutes GI.
FTY720 administration reduced the ischaemia-induced phosphorylation of p38 MAPK (C).
@ P<0.05 vs. Control; n=3-5.

Extracellular signal-regulated kinase p42/p44

FTY720 administered prior to sustained ischaemia failed to elicit any effect on the ischaemia-induced dephosphorylation of ERK p42/p44 (Figure 5.26). The phosphorylation of both ERK p42 and ERK p44 were profoundly reduced by sustained ischaemia, an effect which was unaffected by the administration of FTY720 (ratio of phosphorylated ERK p42 to total ERK p42: Control: 1.00±0.26 AU vs. 20 GI: 0.08±0.01 AU and PreFTY: 0.11±0.04 AU, n=3-5; P<0.01, and ratio of phosphorylated ERK p44 to total ERK p44: Control: 1.00±0.29 AU vs. 20 GI: 0.05±0.02 AU and PreFTY: 0.06±0.02 AU, n=3-5; P<0.01). This implies that PP2A plays no role in the ischaemia-mediated dephosphorylation of ERK p42/p44.

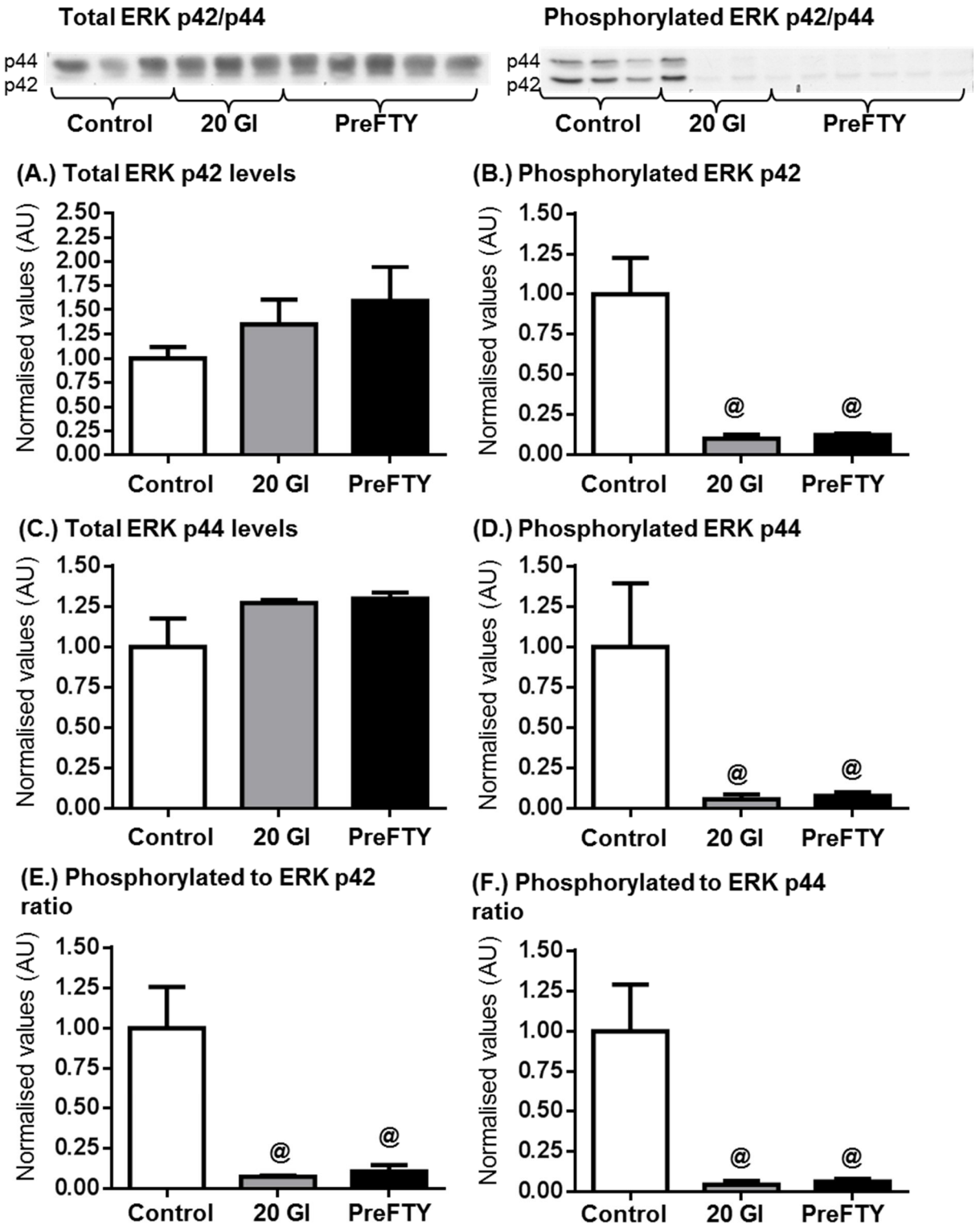


Figure 5.26. ERK p42/p44 at 20 minutes GI following the pre-ischaemic administration of FTY720.
FTY720 failed to elict any effect on ERK p42/p44 levels or the phosphorylation of ERK p42/p44. @: P<0.05 vs Control; n=3-5.

Equal loading: β-Tubulin

Blotting for β-Tubulin revealed no differences between any of the groups, thereby confirming equal loading (Figure 5.27).

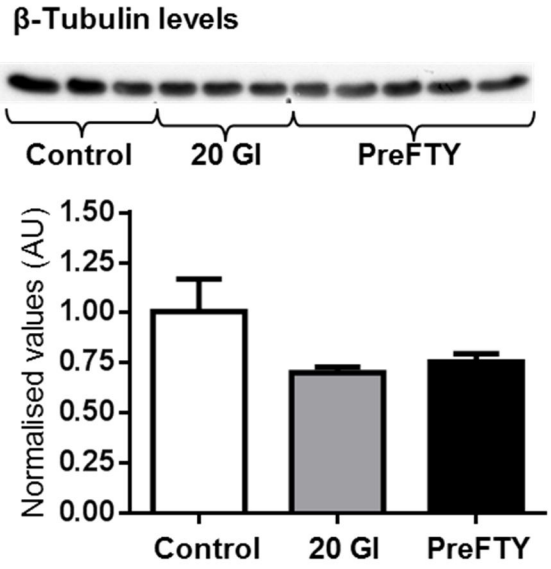


Figure 5.27. Loading control for the effects of pre-ischaemic administered FTY720 at 20 minutes GI. β-Tubulin was used as loading control for the determination of the kinase profiles associated with FTY720 treatment. n=3-5.

Pre-treatment with FTY720: Protein profiles at 5 minutes reperfusion

Perfusion with FTY720 was followed by 20 minutes GI and 5 minutes reperfusion. Heart samples were then flash frozen and analysed using Western blotting techniques.

Protein phosphatase 2A

At 5 minutes reperfusion following 20 minutes GI, pre-ischaemic treatment with FTY720 failed to elicit any effect on the levels of PP2A-C, PP2A-A, or the ratio between the two (Figure 5.28).

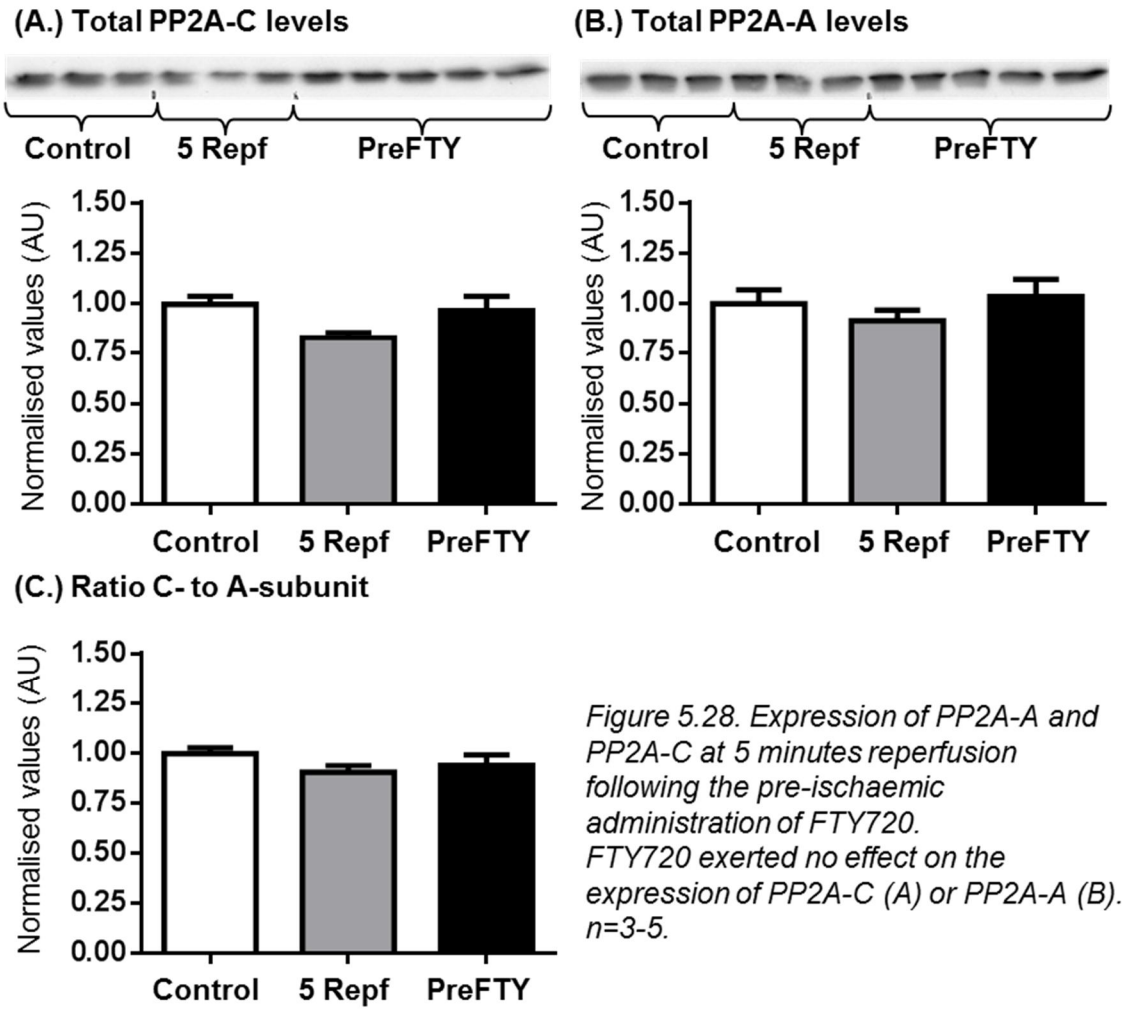


Figure 5.28. Expression of PP2A-A and PP2A-C at 5 minutes reperfusion following the pre-ischaemic administration of FTY720. FTY720 exerted no effect on the expression of PP2A-C (A) or PP2A-A (B). n=3-5.

Concerning the posttranslational modification of PP2A-C (Figure 5.29), FTY720 treatment was associated with an increase in absolute phosphorylation of PP2A-C relative to control (Control: 1.00±0.12 AU vs. PreFTY720: 1.79±0.18 AU, n=3-5; $P<0.05$). Expression of phosphorylated PP2A-C relative to total PP2A-C however revealed that FTY720 did not exert much of an effect in addition to the reperfusion mediated increase in the degree of PP2A-C phosphorylation (Control: 1.00±0.13 AU vs. 5 Repf: 1.75±0.09 AU and PreFTY: 1.84±0.08 AU, n=3-5; $P<0.01$). FTY720 also failed to induce any changes in the methylation of PP2A-C relative to 5 minutes reperfusion alone (nonmethylation relative to total PP2A-C: Control: 1.00±0.08 AU vs. 5 Repf: 1.62±0.12 AU and PreFTY: 1.54±0.07 AU, n=3-4; $P<0.01$). Pre-ischaemic treatment with FTY720 therefore failed to exert any effect on PP2A at 5 minutes reperfusion.

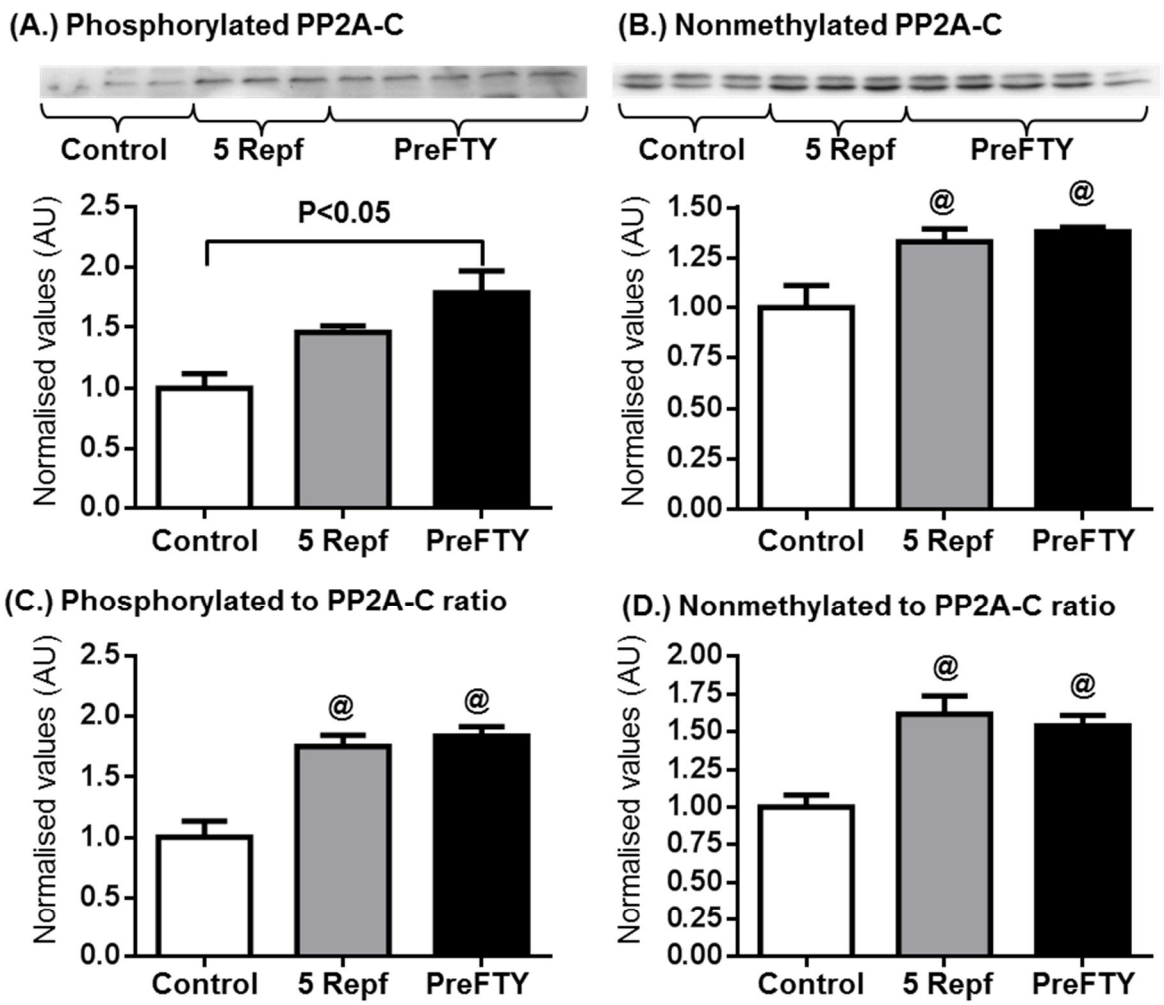


Figure 5.29. Phosphorylation and methylation of PP2A-C in hearts treated with FTY720 prior to 20 minutes GI followed by 5 minutes reperfusion.
Pretreatment with FTY720 increased the absolute phosphorylation of PP2A-C relative to control (A). It however failed to exert any effect on methylation (B&D). @ P<0.05 vs. Control; n=3-5.

Protein kinase B (Akt)

Despite the lack of FTY720 induced effects on PP2A at 5 minutes reperfusion, pretreatment with FTY720 exerted an intriguing effect on PKB/Akt (Figure 5.30). Since absolute phosphorylation and the relative degree of phosphorylation followed the same pattern, I shall only report on the ratio of phosphorylated PKB/Akt relative to total PKB/Akt. Five minutes reperfusion was associated with a pronounced increase in the phosphorylation of PKB/Akt relative to control, irrespective of FTY720 pretreatment (Control: 1.00±0.20 AU vs. 5 Repf: 7.51±0.44 AU and PreFTY: 3.19±0.17 AU, n=3-5; P<0.001). That being noted, pre-ischaemic treatment with FTY720 was associated with a reduction in the phosphorylation of PKB/Akt relative to 5 minutes reperfusion alone (5 Repf: 7.51±0.44 AU vs. PreFTY: 3.19±0.17 AU, n=3-5; P<0.001). FTY720 therefore either down-regulated upstream activating events in the PKB/Akt pathway or directly mediated the dephosphorylation of PKB/Akt. Both these scenarios could accomodate FTY720-mediated activation of PP2A as part of this signal modification.

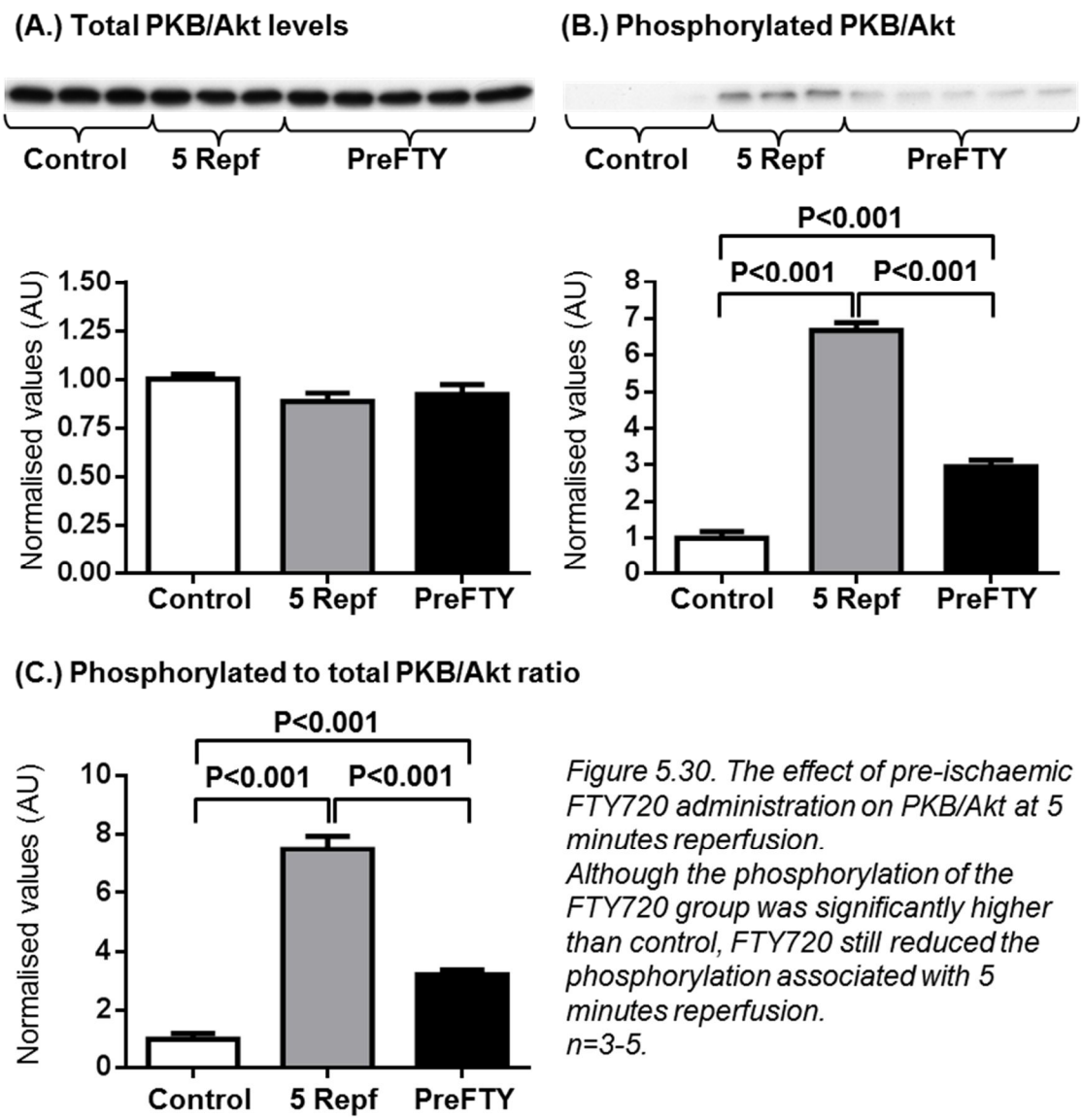


Figure 5.30. The effect of pre-ischaemic FTY720 administration on PKB/Akt at 5 minutes reperfusion.
Although the phosphorylation of the FTY720 group was significantly higher than control, FTY720 still reduced the phosphorylation associated with 5 minutes reperfusion.
n=3-5.

Glycogen synthase kinase-3β

These outspoken effects of pre-ischaemic administered FTY720 on PKB/Akt however failed to induce significant effects on one of the downstream effectors of PKB/Akt signalling, namely GSK-3β (Figure 5.31). The phosphorylation of GSK-3β was increased at 5 minutes reperfusion relative to control, irrespective of FTY720 administration (ratio phosphorylated to total GSK-3β: Control: 1.00±0.09 AU vs. 5 Repf: 8.37±1.81 AU and PreFTY: 5.91±0.85 AU, n=3-5; P<0.05, absolute phosphorylation followed the same pattern).

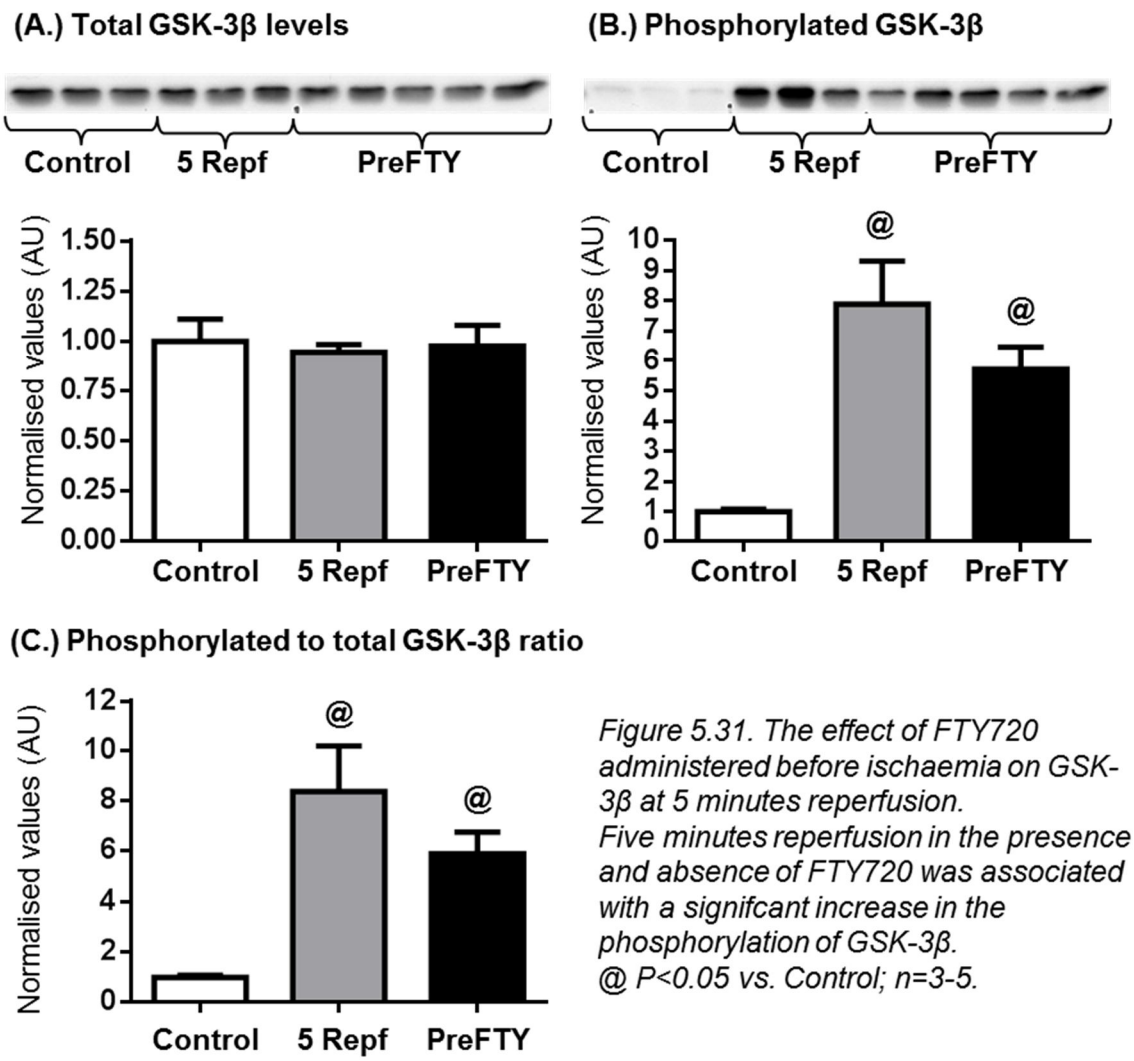


Figure 5.31. The effect of FTY720 administered before ischaemia on GSK-3β at 5 minutes reperfusion.
Five minutes reperfusion in the presence and absence of FTY720 was associated with a signifcant increase in the phosphorylation of GSK-3β.
@ P<0.05 vs. Control; n=3-5.

p38 Mitogen activated protein kinase

Similar to GSK-3β, 5 minutes reperfusion was associated with a profound increase in phosphorylation of p38 MAPK, which was not influenced at all by the pre-ischaemic administration of FTY720 (Figure 5.32: ratio phosphorylated to total p38 MAPK: Control: 1.00±0.38 AU vs. 5 Repf: 6.47±1.01 AU and PreFTY: 7.86±1.03 AU, n=3-5; P<0.05, absolute phosphorylation followed the same pattern). It therefore seems that at this time point p38 MAPK is unaffected by the signalling alterations associated with FTY720.

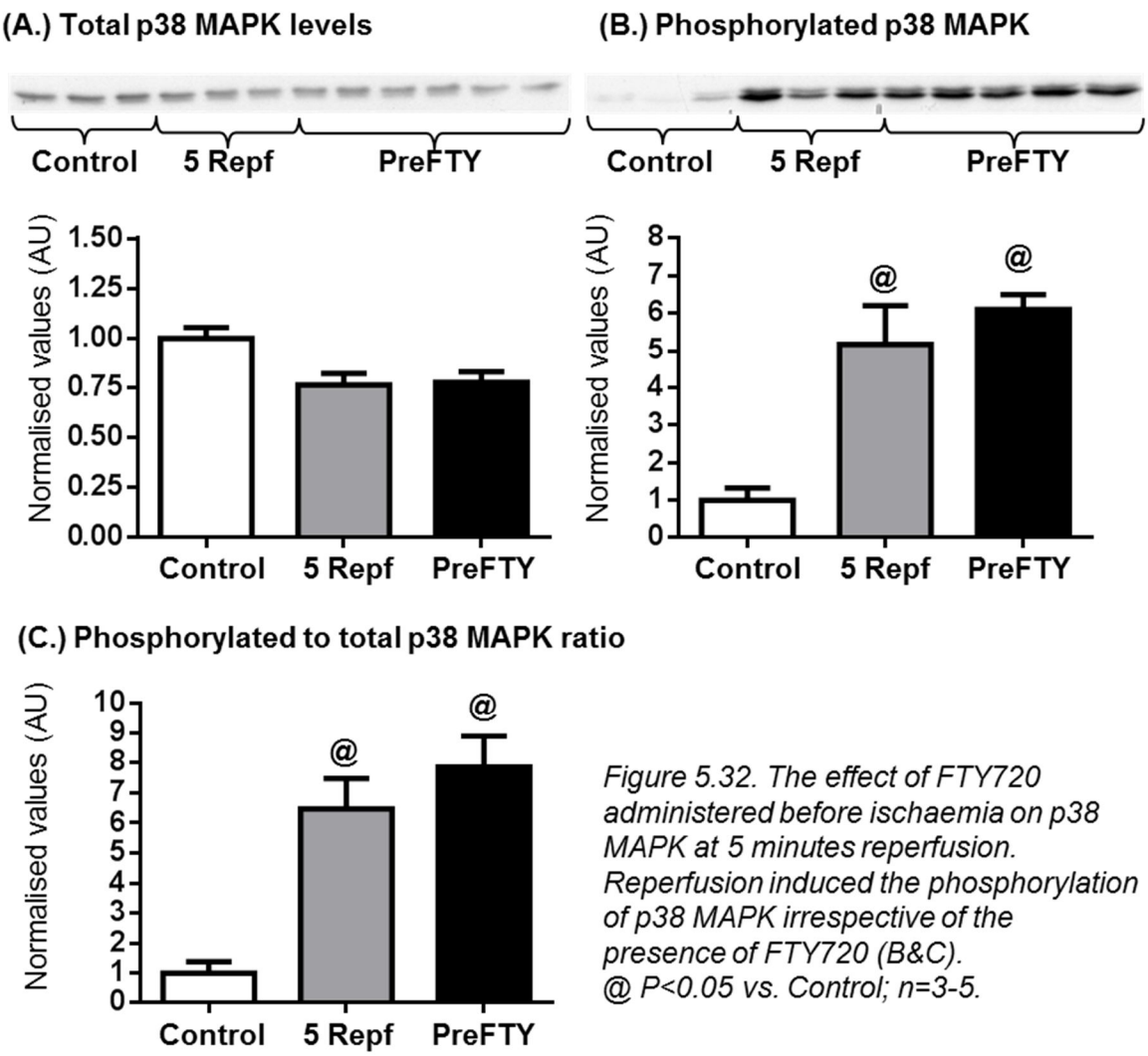


Figure 5.32. The effect of FTY720 administered before ischaemia on p38 MAPK at 5 minutes reperfusion. Reperfusion induced the phosphorylation of p38 MAPK irrespective of the presence of FTY720 (B&C). @ P<0.05 vs. Control; n=3-5.

Extracellular signal-regulated kinase p42/p44

At 5 minutes reperfusion pre-ischaemic FTY720 treatment was associated with an increase in the phosphorylation of ERK p42/p44 relative to total ERK p42/44 in comparison to control (Figure 5.33: ERK p42: Control: 1.00±0.54 AU vs. PreFTY: 8.03±1.52 AU, n=3-5; P<0.05, and ERK p44: Control: 1.00±0.42 AU vs. PreFTY: 5.43±0.97 AU, n=3-5; P<0.05). Surprisingly, 5 minutes reperfusion alone was not associated with a significant increase in the phosphorylation of ERK p42/44, despite an almost obvious visible increase in ERK p42/p44 phosphorylation according to the blot. The lack of statistical significance can be attributed to the unfortunate degree of variation within the data combined with low n-values for these groups (ERK p42: Control: 1.00±0.54 AU vs. 5 Repf: 3.24±0.59 AU, n=3; NS, and ERK p44: Control: 1.00±0.42 AU vs. 5 Repf: 2.75±0.73 AU, n=3; NS).

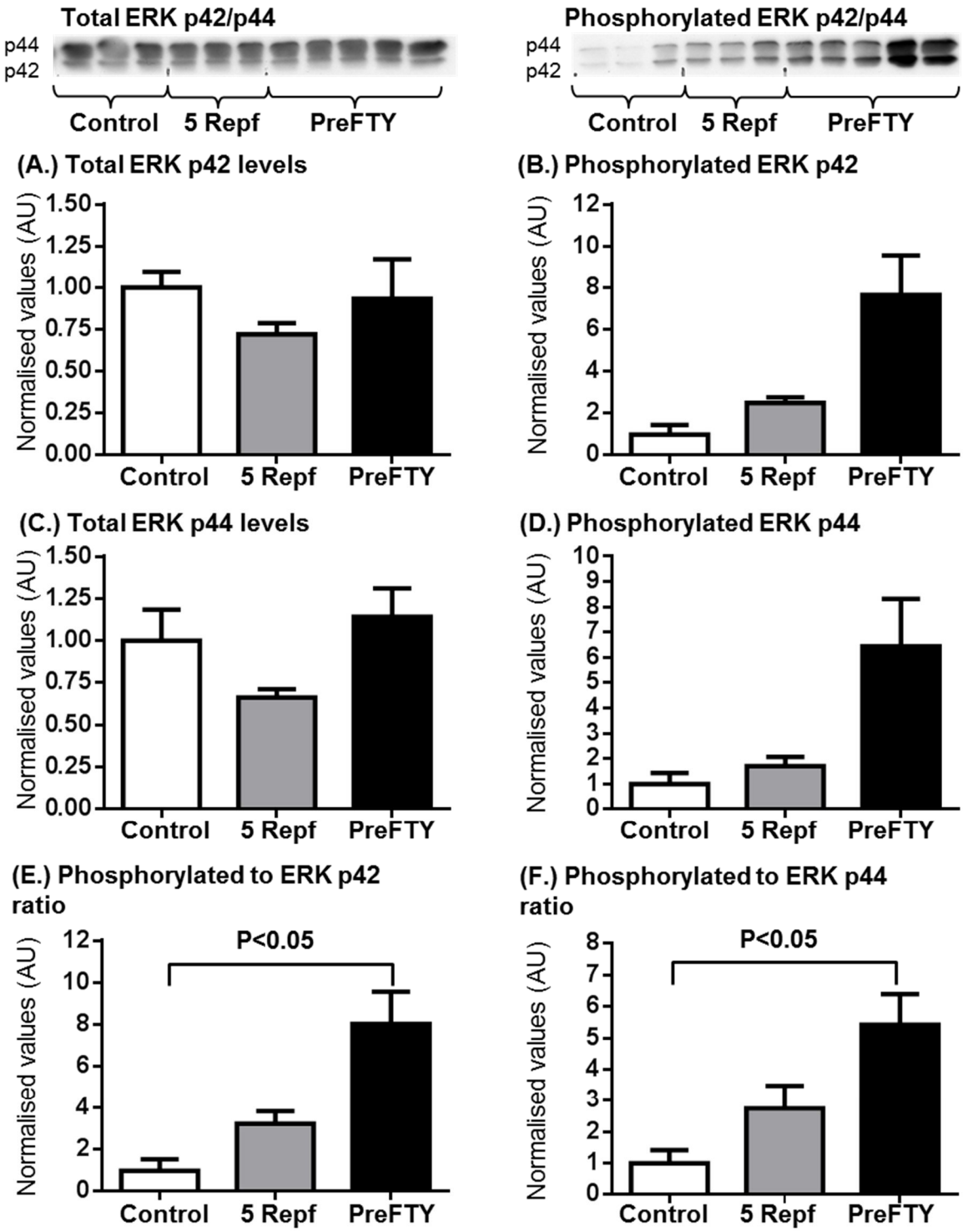


Figure 5.33. ERK p42/p44 at 5 minutes reperfusion following the pre-ischaemic administration of FTY720.
Pre-ischaemic administration of FTY720 was associated with an increase in the phosphorylation of both ERK p42 and p44 compared to control. n=3-5.

Equal loading: β-Tubulin

Western blotting of a representative blot for β-tubulin confirmed equal loading (Figure 5.34).

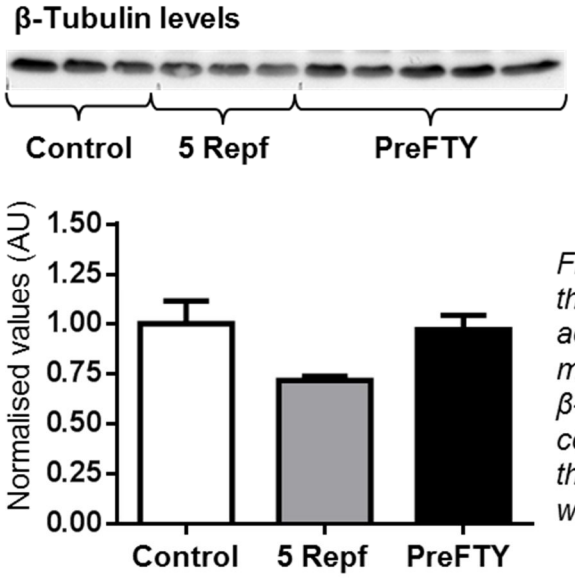


Figure 5.34. Loading control for the effects of pre-ischaemic administered FTY720 at 5 minutes reperfusion. β-Tubulin was used as loading control for the determination of the kinase profiles associated with FTY720 treatment. n=3-5.

Pre-treatment with FTY720: Protein profiles at 10 minutes reperfusion

Hearts exposed to 15 minutes perfusion with 1 μM FTY720 were immediately exposed to 20 minutes ischaemia, followed by 10 minutes reperfusion. Hearts were then freeze-clamped and the selected signalling proteins investigated using Western blotting.

Protein phosphatase 2A

Pre-ischaemic treatment with FTY720 did not lead to any changes in the levels of either PP2A-C or PP2A-A (Figure 5.35).

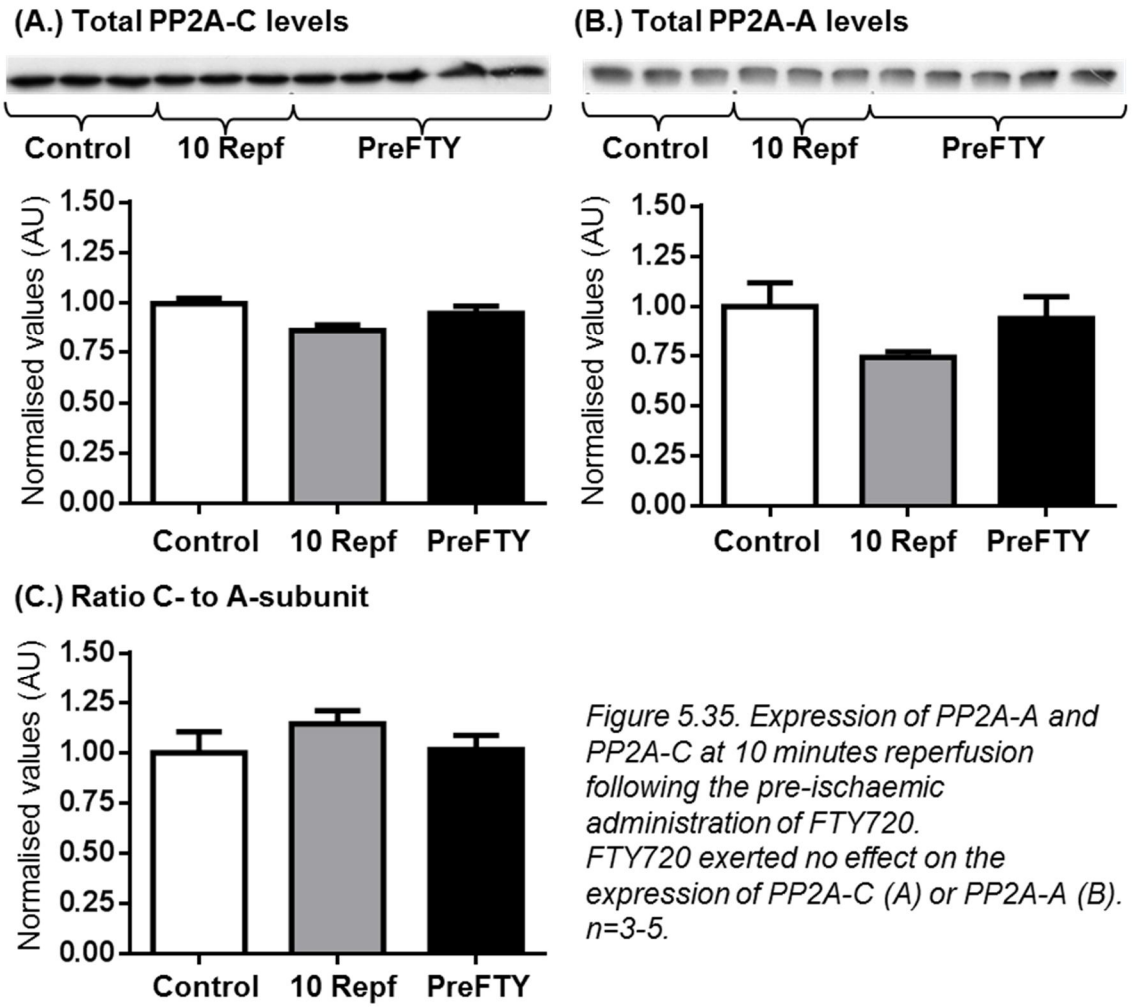


Figure 5.35. Expression of PP2A-A and PP2A-C at 10 minutes reperfusion following the pre-ischaemic administration of FTY720. FTY720 exerted no effect on the expression of PP2A-C (A) or PP2A-A (B). n=3-5.

Interestingly, FTY720 treatment elicited an increase in the phosphorylation of PP2A-C, but only in comparison to control (Figure 5.36: ratio phosphorylated to total PP2A-C: Control: 1.00±0.11 AU vs. PreFTY: 1.57±0.09 AU, n=3-5; $P<0.05$) and not 10 minutes reperfusion on its own (10 Repf: 1.55±0.16 AU vs. PreFTY: 1.57±0.09 AU, n=3-5; NS). This indicates that the increased phosphorylation associated with FTY720 treatment is not very strong, especially in comparison to 10 minutes reperfusion alone, and therefore probably not important. Concerning the methylation of PP2A-C, pre-ischaemic FTY720 treatment also failed to induce any changes relative to 10 minutes reperfusion. It can therefore be concluded that, just as at 5 minutes reperfusion, FTY720 exerts no effects on PP2A at 10 minutes reperfusion. The FTY720 mediated activation of PP2A is therefore transient and does not extend beyond 20 minutes GI.

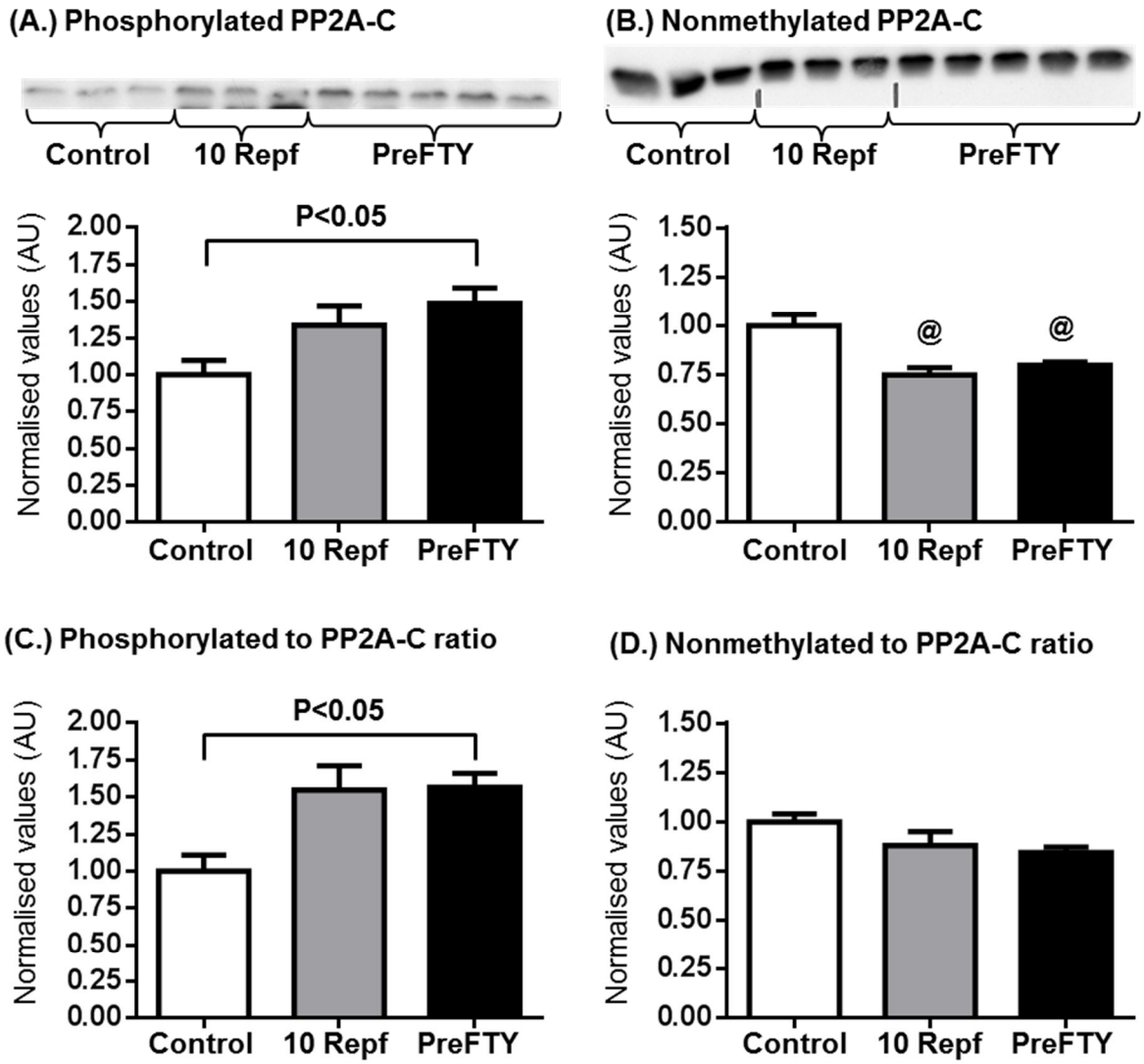


Figure 5.36. Phosphorylation and methylation of PP2A-C in hearts treated with FTY720 prior to 20 minutes GI followed by 10 minutes reperfusion.
Pretreatment with FTY720 increased the phosphorylation of PP2A-C relative to control (A&C). It however failed to exert any effect on methylation (B&D). @ P<0.05 vs. Control; n=3-5.

Protein kinase B (Akt)

The outspoken effects of reperfusion and FTY720 treatment on PKB/Akt phosphorylation which was evident at 5 minutes reperfusion (Figure 5.30), was no longer present after 10 minutes reperfusion (Figure 5.37). An unpaired T-test comparing control with 10 minutes reperfusion reveals that PKB/Akt phosphorylation was still elevated at this time point (phosphorylated to total PKB/Akt ratio: Control: 1.00±0.04 AU vs. 10 Repf: 2.00±0.10 AU, n=3; P<0.001). Although PreFTY was associated with a significant increase in PKB/Akt phosphorylation relative to control (ratio phosphorylated to total PKB/Akt: Control: 1.00±0.04 AU vs. PreFTY: 3.03±0.52 AU, n=3-5; P<0.05), it did not differ significantly from the group exposed to 10 minutes reperfusion alone, indicating that this was not a profound increase in phosphorylation.

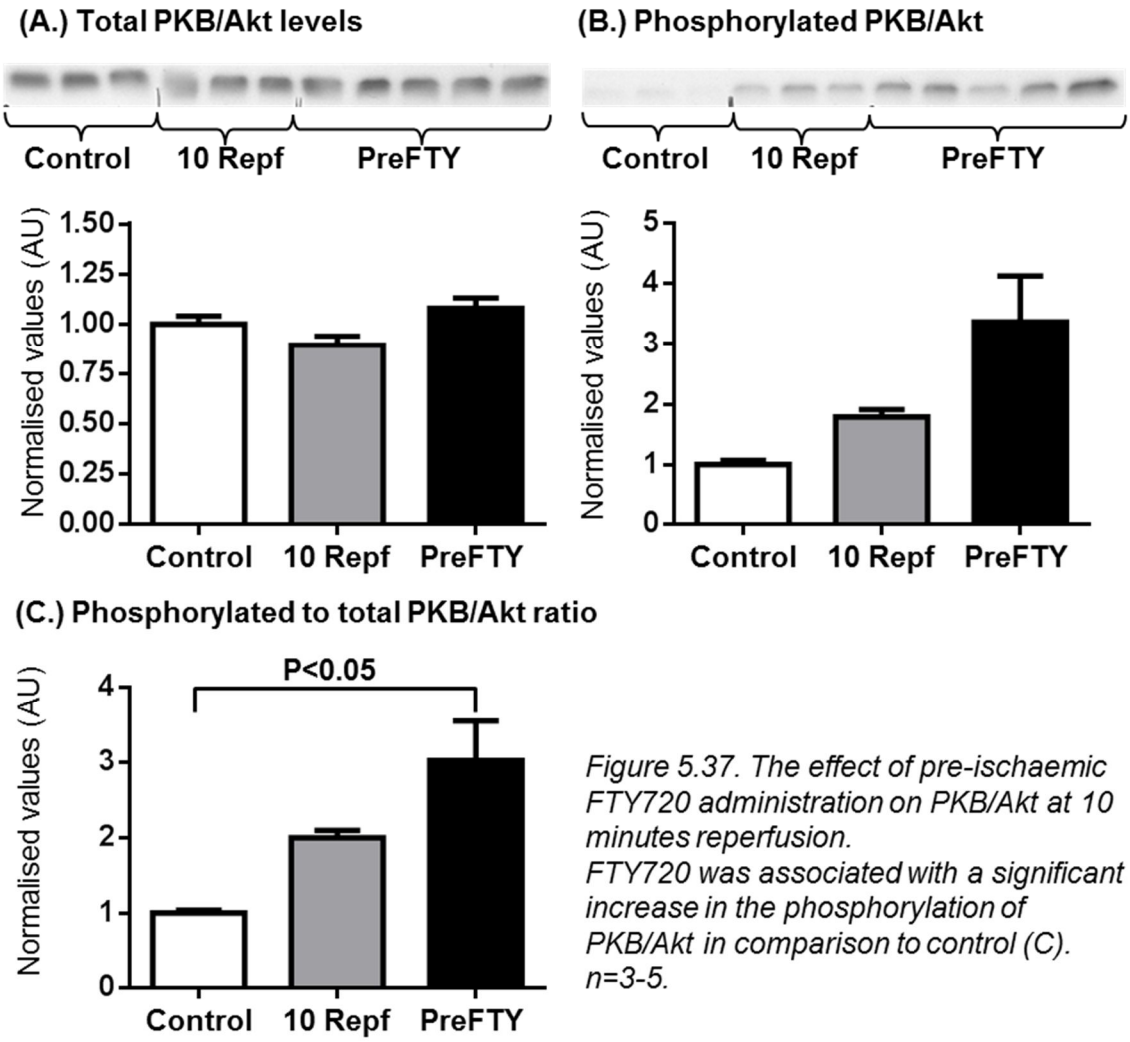


Figure 5.37. The effect of pre-ischaemic FTY720 administration on PKB/Akt at 10 minutes reperfusion.
FTY720 was associated with a significant increase in the phosphorylation of PKB/Akt in comparison to control (C). n=3-5.

Glycogen synthase kinase-3β

Despite the borderline stimulatory effect of FTY720 on PKB/Akt (Figure 5.37), it was definitely associated with an outspoken increase in the phosphorylation of GSK-3β (Figure 5.38) relative to both control (ratio phosphorylated to total GSK-3β: Control: 1.00±0.12 AU vs. PreFTY: 11.19±0.25 AU, n=3-5; P<0.0001), as well as 10 minutes reperfusion (ratio phosphorylated to total GSK-3β: 10 Repf: 5.83±0.83 AU vs. PreFTY: 11.19±0.25 AU, n=3-5; P<0.001). This increase in GSK-3β is all the more impressive since it is in addition to the increase in phosphorylation already induced by reperfusion itself (ratio phosphorylated to total GSK-3β: Control: 1.00±0.12 AU vs. 10 Repf: 5.83±0.83 AU, n=3; P<0.001). Pre-ischaemic treatment with FTY720 therefore elicited a surprising and sudden increase in the phosphorylation of GSK-3β at 10 minutes reperfusion, over and above the change induced by 10 minutes reperfusion alone.

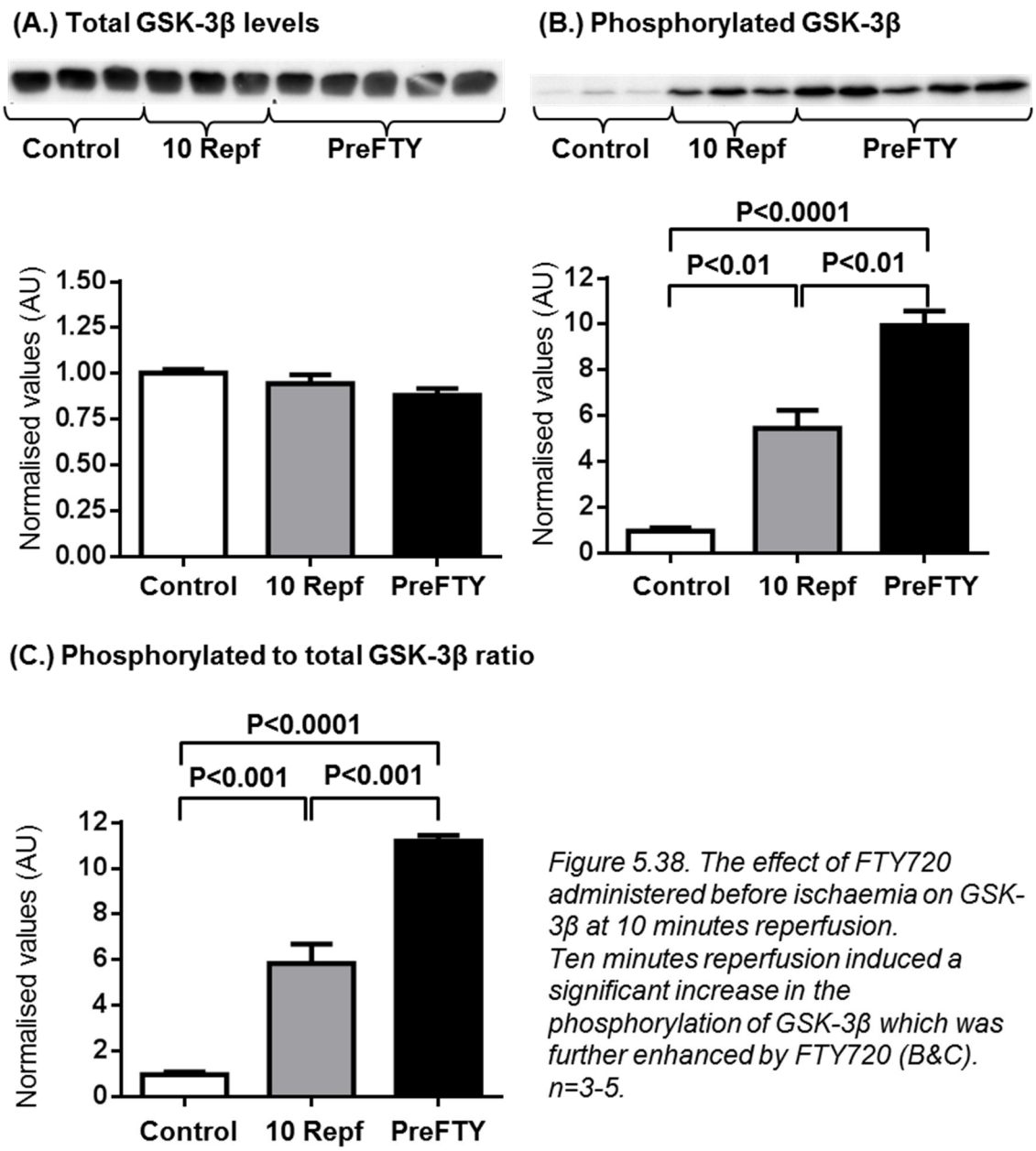


Figure 5.38. The effect of FTY720 administered before ischaemia on GSK-3β at 10 minutes reperfusion. Ten minutes reperfusion induced a significant increase in the phosphorylation of GSK-3β which was further enhanced by FTY720 (B&C). n=3-5.

p38 Mitogen activated protein kinase

As shown in Figure 5.39, 10 minutes reperfusion was associated with an increase in the phosphorylation of p38 MAPK, with pre-ischaemic FTY720 administration exerting no effect on this (ratio of phosphorylated to total p38 MAPK: Control: 1.00±0.34 AU vs. 10 Repf: 5.79±0.92 AU and PreFTY: 6.75±0.43 AU, n=3-5; P<0.01). The effects of FTY720 on p38 MAPK therefore seems to have dissipated by 10 minutes reperfusion.

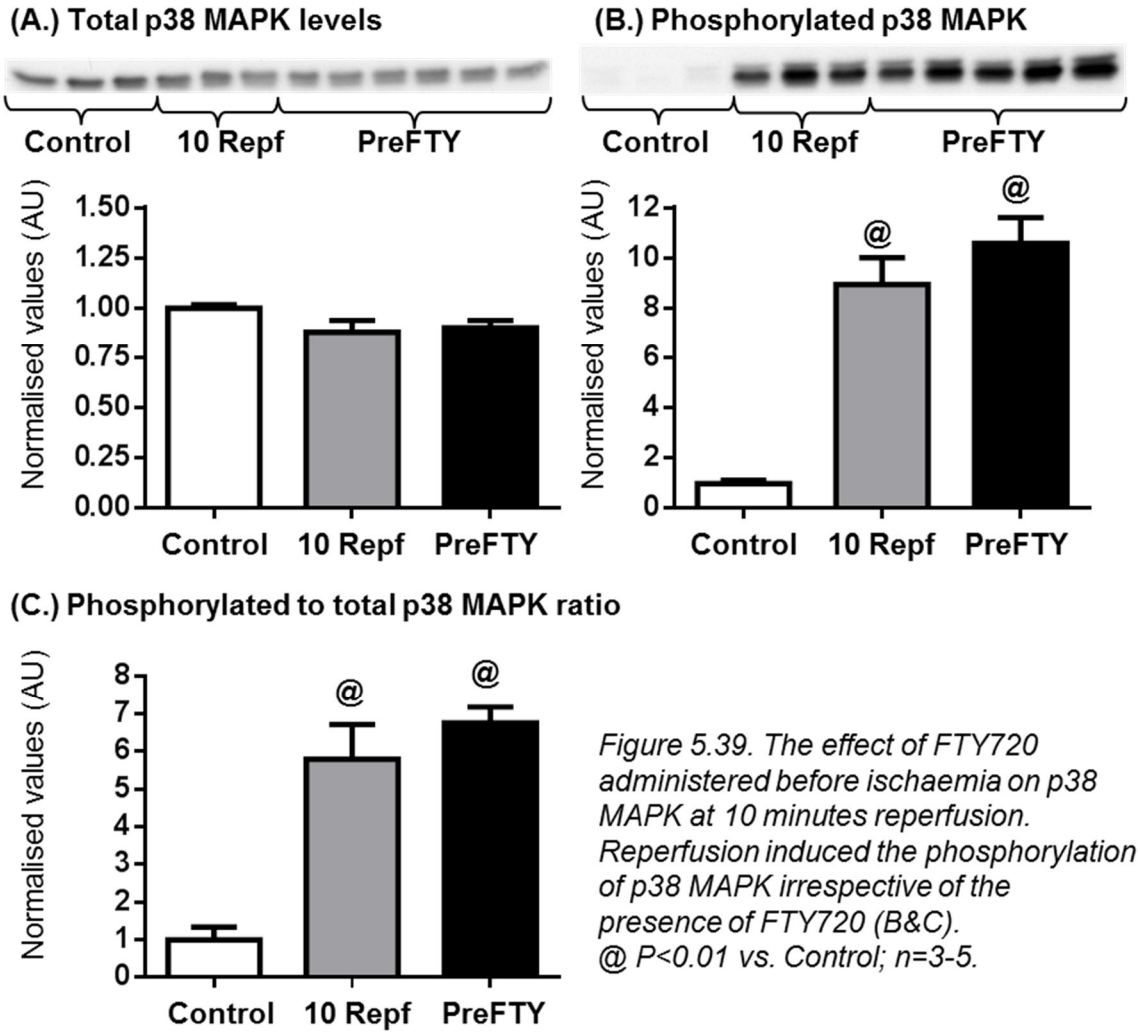


Figure 5.39. The effect of FTY720 administered before ischaemia on p38 MAPK at 10 minutes reperfusion. Reperfusion induced the phosphorylation of p38 MAPK irrespective of the presence of FTY720 (B&C). @ P<0.01 vs. Control; n=3-5.

Extracellular signal-regulated kinase p42/p44

Following a similar pattern to 5 minutes reperfusion, pre-ischaemic FTY720 was associated with an increase in the phosphorylation of ERK p42/p44 (Figure 5.40). In the case of ERK p42 this increase was only relative to control (ratio of phosphorylated to total ERK p42: Control: 1.00±0.33 AU vs. PreFTY: 2.12±0.19 AU, n=3-5; P<0.05), while for ERK p44 it was also elevated compared to 10 minutes reperfusion (ratio of phosphorylated to total ERK p44: Control: 1.00±0.29 AU and 10 Repf: 1.56±0.19 AU vs. PreFTY: 4.60±0.66 AU, n=3-5; P<0.01). Although 10 minutes reperfusion itself was not associated with an increase in the phosphorylation of ERK p42 in comparison to control, a direct comparison reveals a significant increase in the phosphorylation of ERK p42 at 10 minutes reperfusion *per se* (Control: 1.00±0.33 AU vs. 10 Repf: 1.99±0.09 AU, n=3; T-test: P<0.05). The data indicate that FTY720 enhances the phoshorylation already induced by 10 minutes reperfusion itself.

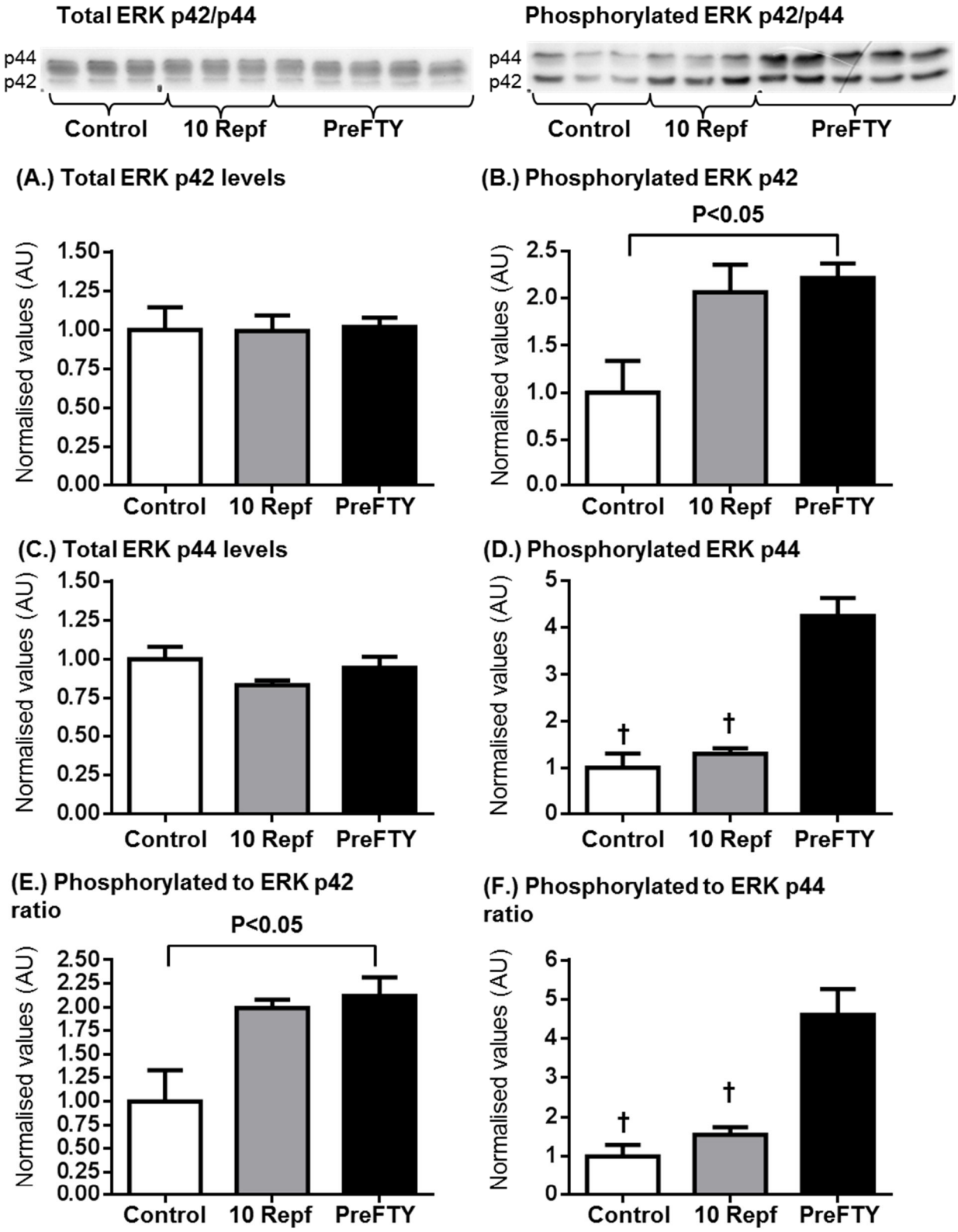


Figure 5.40. ERK p42/p44 at 10 minutes reperfusion following the pre-ischaemic administration of FTY720.
Pre-ischaemic administration of FTY720 enhanced the phosphorylation of ERK p42 compared to control (B&E) and even compared to 10 minutes reperfusion for ERK p44 (D&F).
† P<0.01 vs. PreFTY; n=3-5.

Equal loading: β-Tubulin

Western blotting of a representative blot for β-tubulin showed that there were no statistical differences between any of the groups (Figure 5.41).

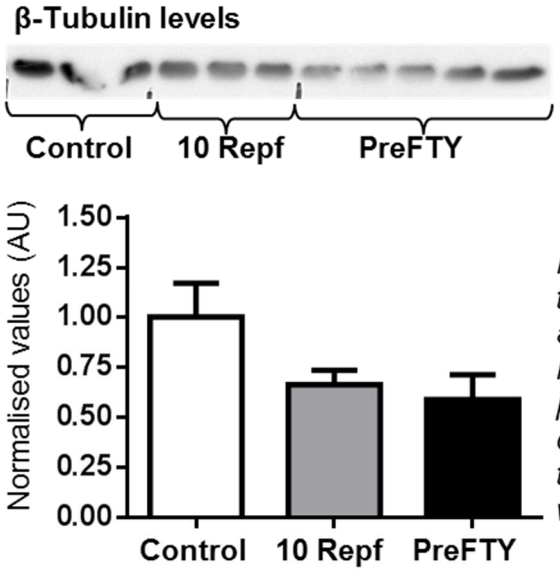


Figure 5.41. Loading control for the effects of pre-ischaemic administered FTY720 at 10 minutes reperfusion. β-Tubulin was used as loading control for the determination of the kinase profiles associated with FTY720 treatment. n=3-5.

Reperfusion-treatment with FTY720: Protein profiles at 15 minutes reperfusion

In these experiments hearts were exposed to 20 minutes GI, followed by FTY720 (1 μM) administration at the onset of reperfusion for a period of 15 minutes. At the end of this 15 minute period, hearts were freeze-clamped for subsequent determination of the signalling protein profiles.

Protein phosphatase 2A

Administration of FTY720 at the onset of reperfusion was not associated with any changes in the levels of total PP2A-C or PP2A-A (Figure 5.42). Interestingly, comparison of the ratio between PP2A-C and PP2A-A with control using a Dunnett's *post hoc* test shows that this ratio was increased at 15 minutes reperfusion (Control: 1.00±0.09 AU vs. 15 Repf: 1.21±0.04 AU, n=3; $P<0.05$). This is a very slight increase which, although it fits the trend of an increased ratio at 5 minutes reperfusion (Figure 3.21), is in contrast to the absence of such a difference at 10 minutes reperfusion (Figure 3.22).

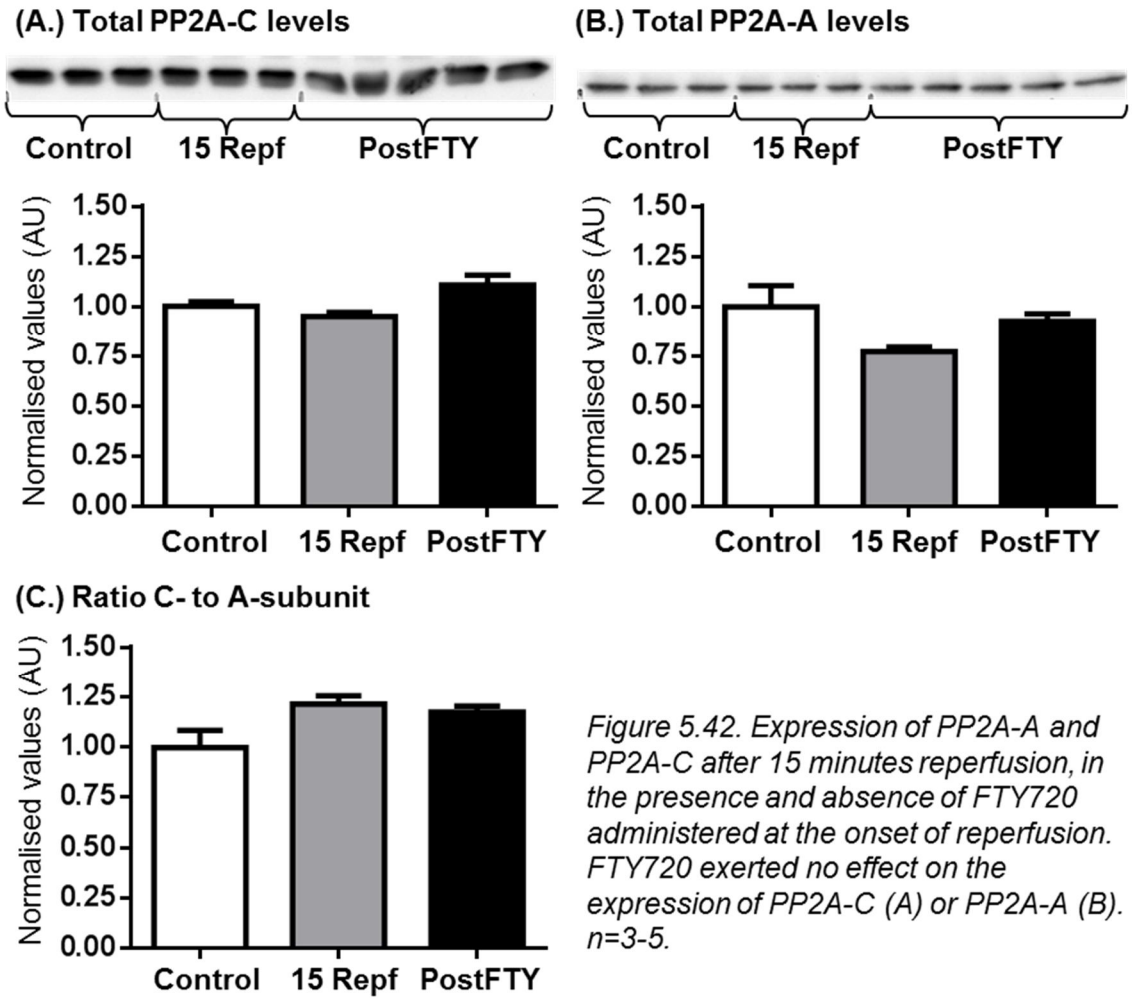


Figure 5.42. Expression of PP2A-A and PP2A-C after 15 minutes reperfusion, in the presence and absence of FTY720 administered at the onset of reperfusion. FTY720 exerted no effect on the expression of PP2A-C (A) or PP2A-A (B). n=3-5.

Surprisingly, reperfusion treatment was not associated with any changes in the phosphorylation of PP2A-C (Figure 5.43). It however elicited a reduction in methylation, but only relative to control (ratio nonmethylated PP2A-C to total PP2A-C: Control: 1.00±0.08 AU vs. PostFTY: 1.22±0.04 AU, n=3-5; $P<0.05$). In the absence of an effect relative to 15 minutes reperfusion only (in the absence of the drug), this observed change probably represents a very slight reduction in PP2A-C methylation. Post-treatment with FTY720 therefore elicited surprisingly little effect on PP2A.

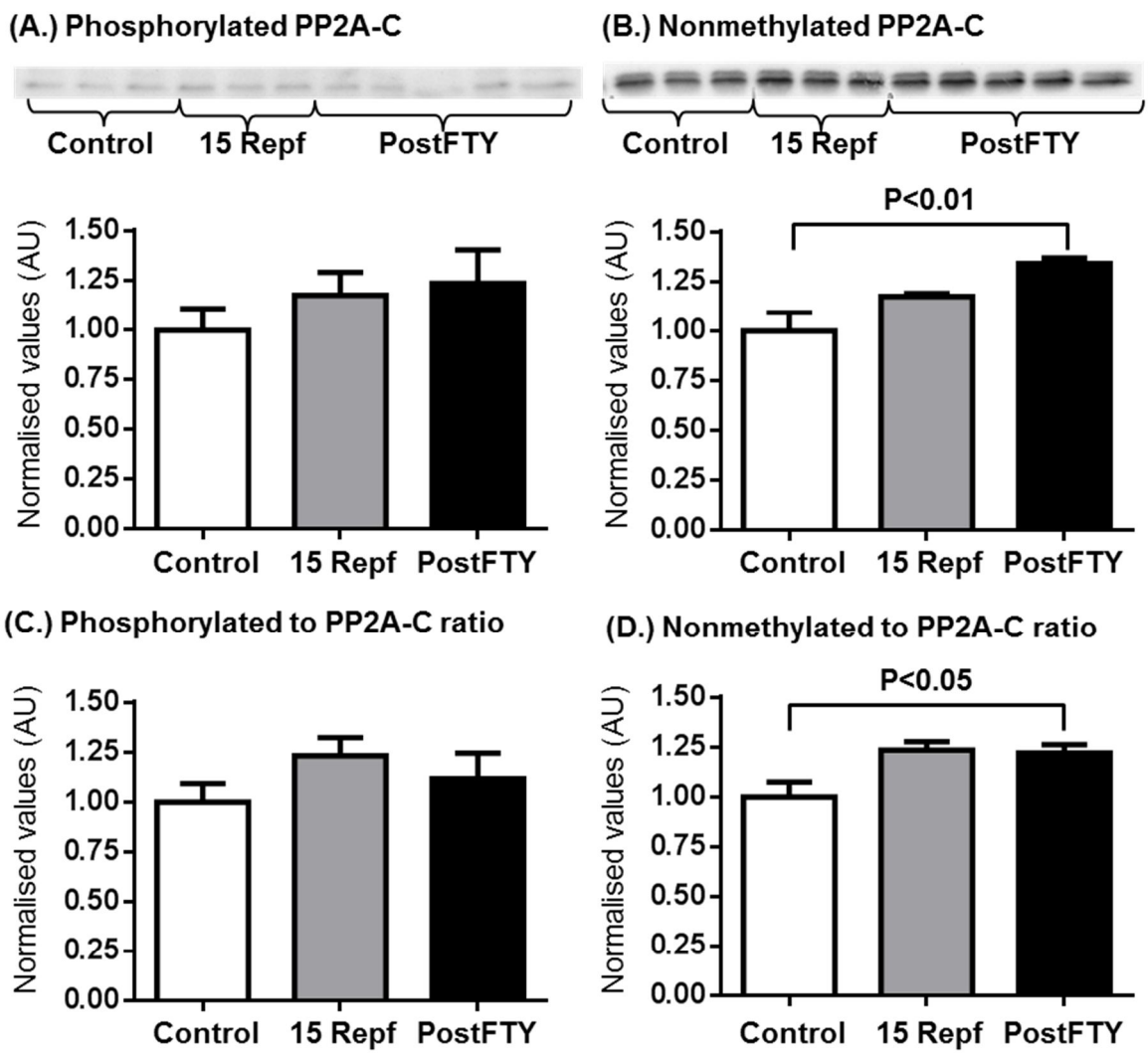


Figure 5.43. Phosphorylation and methylation of PP2A-C in hearts treated with FTY720 following 20 minutes GI at 15 minutes reperfusion.
FTY treatment failed to exert any effect on PP2A phosphorylation (A&C), although it was associated with an increase in the levels of nonmethylated PP2A-C (B&D).
n=3-5.

Protein kinase B (Akt)

Fifteen minutes reperfusion was in itself associated with a profound increase in the phosphorylation of PKB/Akt (Figure 5.44). The administration of FTY720 in combination with reperfusion failed to elicit any effect on this increase in phosphorylation (ratio of phosphorylated to total PKB/Akt: Control: 1.00±0.04 AU vs. 15 Repf: 6.96±0.47 AU and PostFTY: 6.06±0.96 AU, n=3-5; P<0.01).

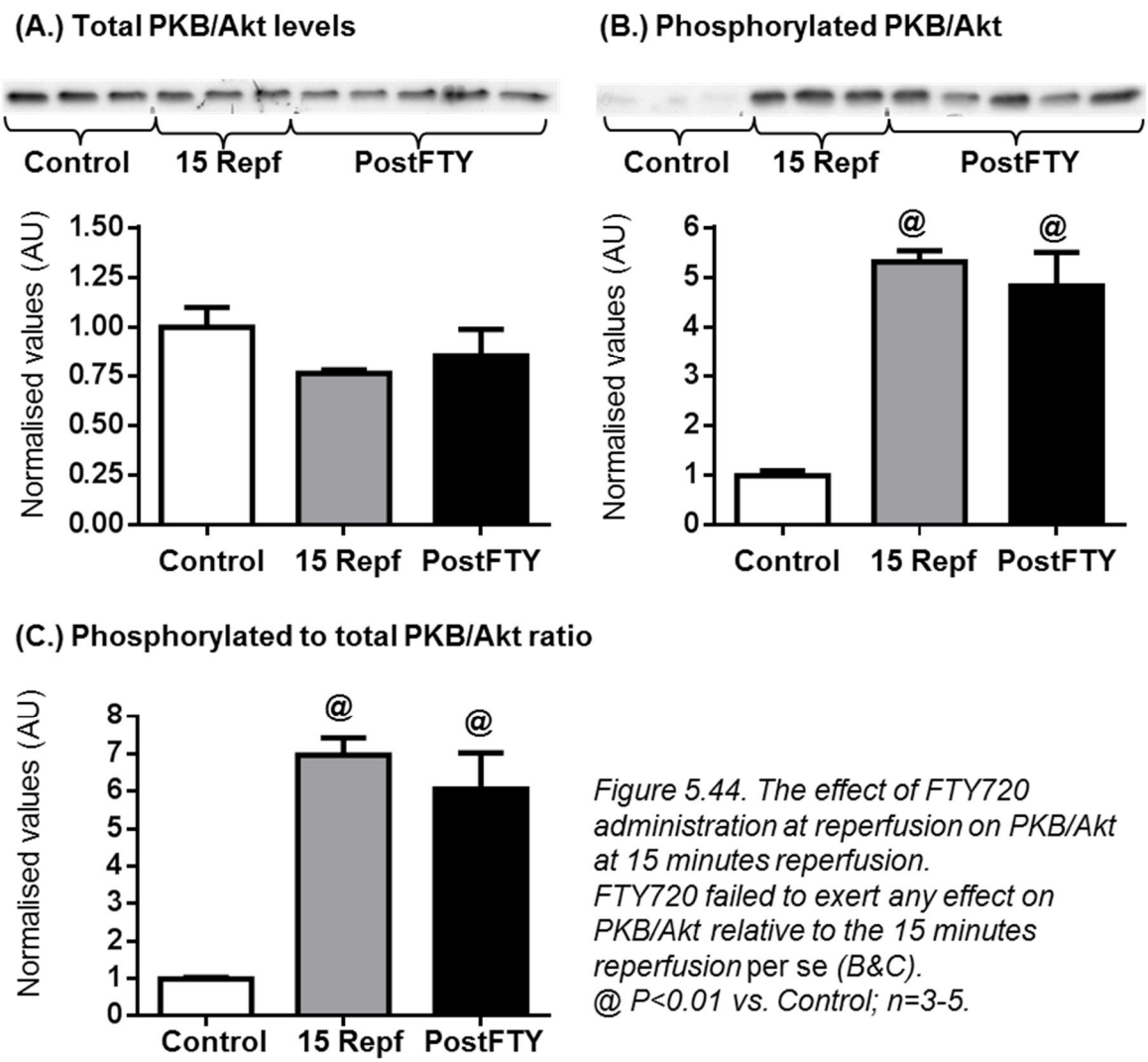


Figure 5.44. The effect of FTY720 administration at reperfusion on PKB/Akt at 15 minutes reperfusion.
FTY720 failed to exert any effect on PKB/Akt relative to the 15 minutes reperfusion per se *(B&C).*
@ P<0.01 vs. Control; n=3-5.

Glycogen synthase kinase-3β

As was seen with PKB/Akt, 15 minutes reperfusion in itself was associated with a potent increase in the phosphorylation of GSK-3β (Figure 5.45). Statistically FTY720 administration failed to impact this increase in phosphorylation (ratio of phosphorylated to total GSK-3β: Control: 1.00±0.11 AU vs. 15 Repf: 6.79±1.22 AU and PostFTY: 4.21±0.58 AU, n=3-5; $P<0.05$). However, a direct comparison between 15 minutes reperfusion alone and the FTY720 treatment group shows a bordeline significant difference between the two groups (ratio of phosphorylated to total GSK-3β: 15 Repf: 6.79±1.22 AU vs. PostFTY: 4.21±0.58 AU, n=3-5; $P=0.07$). Our data therefore reveal a strong trend indicating that FTY720 treatment reduced the reperfusion mediated phosphorylation of GSK-3β.

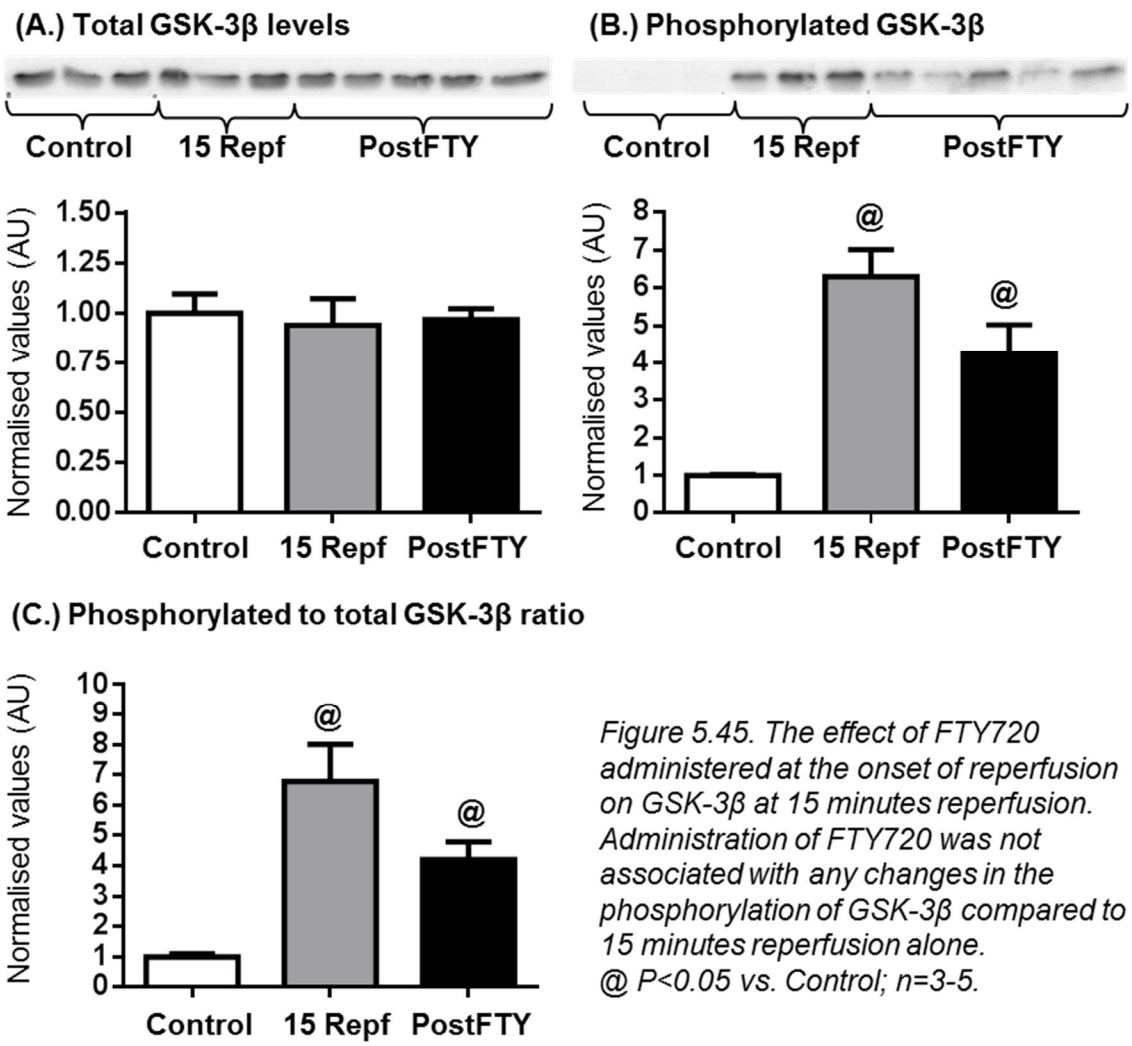


Figure 5.45. The effect of FTY720 administered at the onset of reperfusion on GSK-3β at 15 minutes reperfusion. Administration of FTY720 was not associated with any changes in the phosphorylation of GSK-3β compared to 15 minutes reperfusion alone. @ P<0.05 vs. Control; n=3-5.

p38 Mitogen activated protein kinase

P38 MAPK fell into the same pattern as all the other kinases analysed, in the sense that 15 minutes reperfusion itself already potently activated the kinase (Figure 5.46). As with the other kinases, FTY720 treatment failed to make any difference in this reperfusion-associated elevation in phosphorylation (ratio of phosphorylated to total p38 MAPK: Control: 1.00±0.09 AU vs. 15 Repf: 8.78±1.38 AU and PostFTY: 7.13±10.02 AU, n=3-5; P<0.01).

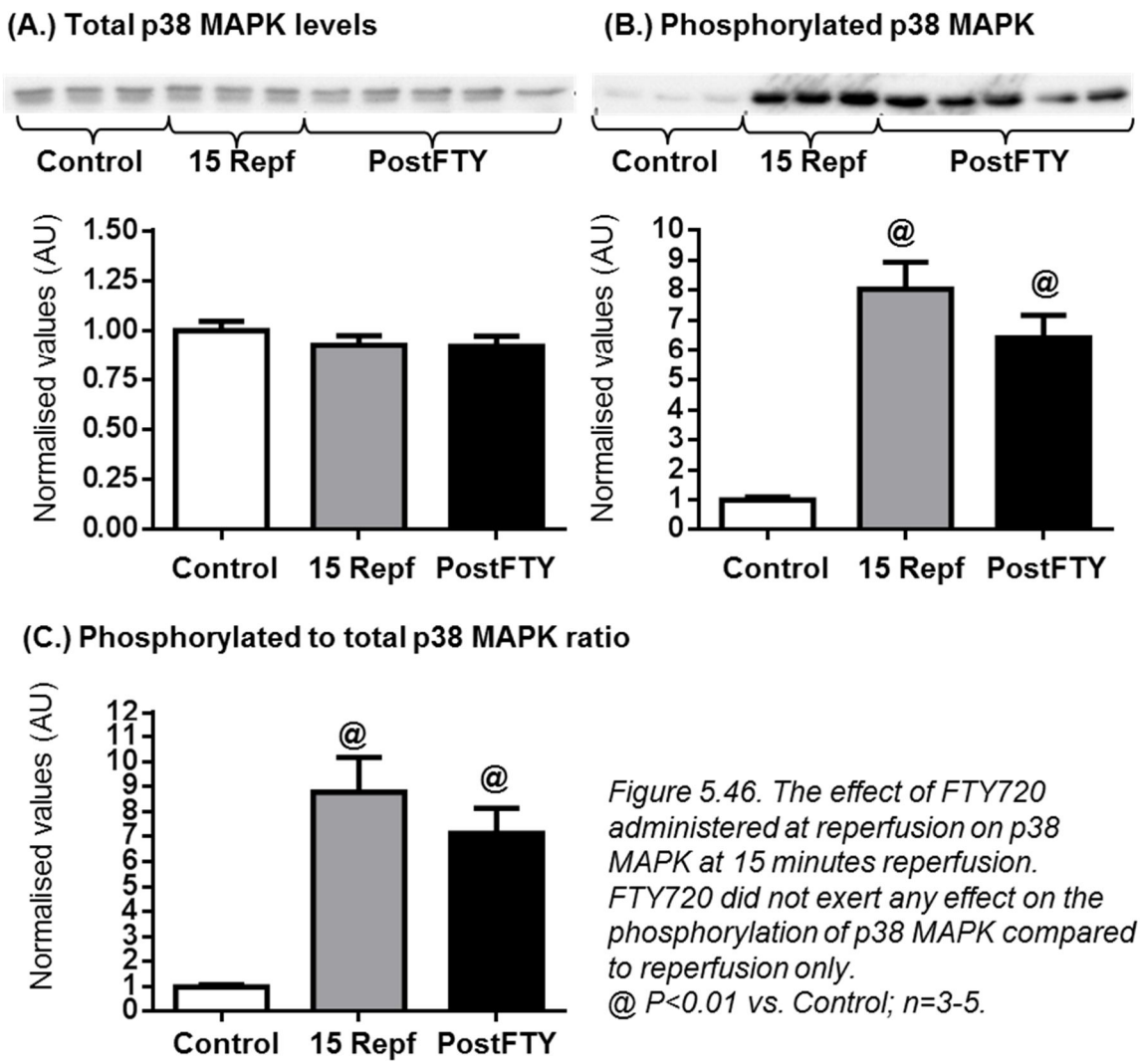


Figure 5.46. The effect of FTY720 administered at reperfusion on p38 MAPK at 15 minutes reperfusion. FTY720 did not exert any effect on the phosphorylation of p38 MAPK compared to reperfusion only. @ P<0.01 vs. Control; n=3-5.

Extracellular signal-regulated kinase p42/p44

FTY720 administered during reperfusion failed to elicit a strong and robust effect on the phosphorylation of ERK p42/p44 (Figure 5.47). Reperfusion itself increased the phosphorylation of ERK p42 and FTY720 failed to impact this at all (ratio phosphorylated to total ERK p42: Control: 1.00±0.22 AU vs. 15 Repf: 2.77±0.49 AU and PostFTY: 2.18±0.07 AU, n=3-5; P<0.05). Interestingly, the effect of reperfusion on ERK p44 was much less pronounced and FTY720 treatment seemed to enhance it, so that FTY720 treatment was associated with a significant increase in phosphorylation relative to control (ratio phosphorylated to total ERK p44: Control: 1.00±0.24 AU vs. PostFTY: 2.21±0.17 AU, n=3-5; P<0.01). This elevation was however not significant in comparison to reperfusion alone. Direct comparison of control with reperfusion alone reveals a borderline significant difference (ratio phosphorylated to total ERK p44: Control: 1.00±0.24 AU vs. 15 Repf: 1.68±0.21 AU, n=3; T-test: P=0.0968). The absence of a statistical difference (P<0.05) can possibly be attributed to the low n-value. Taken together, FTY720 failed to elicit any outspoken effects on ERK p42/p44 activation when administered during reperfusion.

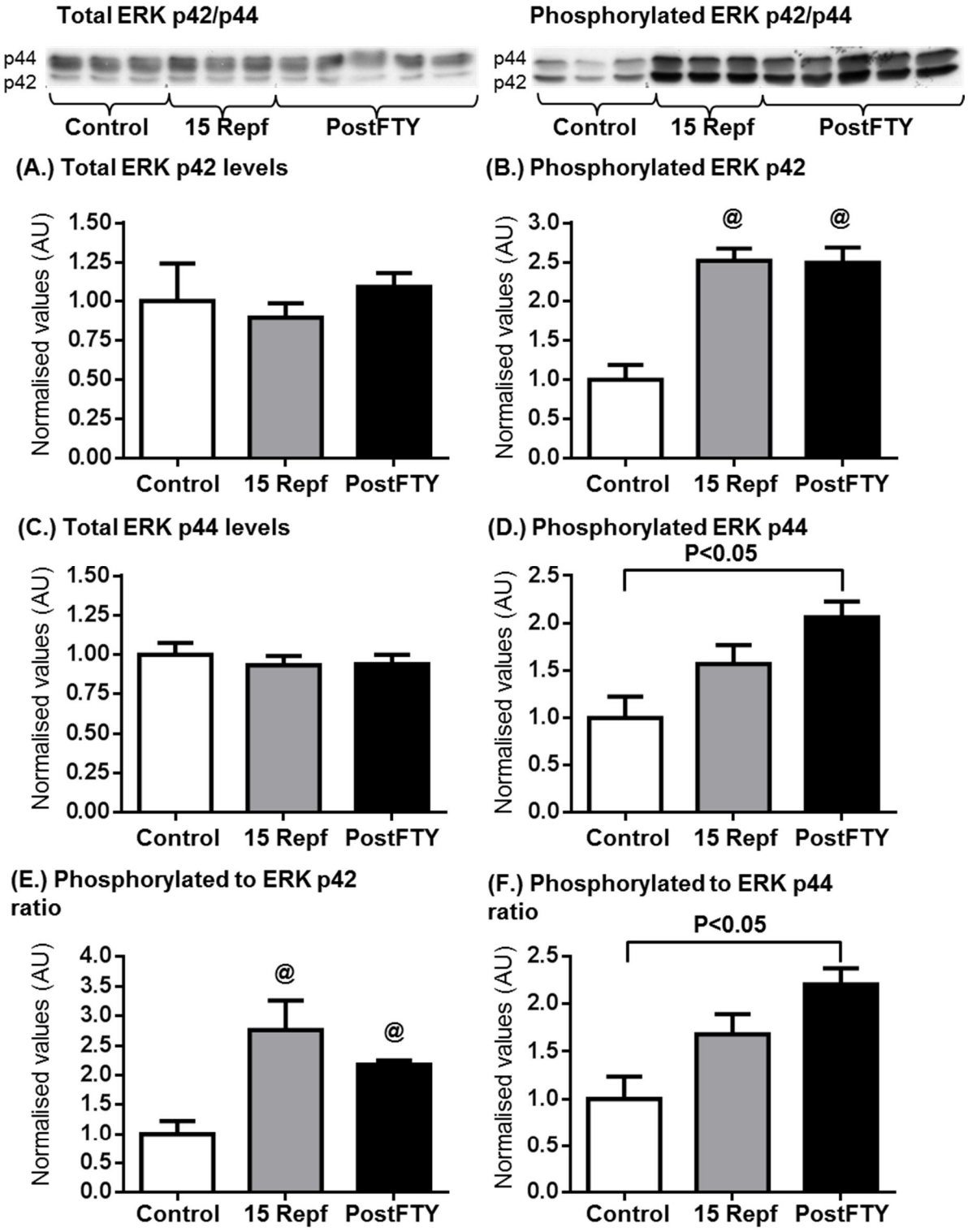


Figure 5.47. ERK p42/p44 at 15 minutes reperfusion following the reperfusion administration of FTY720.
FTY720 administration was associated with an enhancement of the phosphorylation of specifically ERK p44 (D&F), while it exerted no effect on the reperfusion induced phosphorylation of ERK p42.
@ P<0.05 vs. Control; n=3-5.

Equal loading: β-Tubulin

Western blotting for β-tubulin on a representative blot failed to show any significant differences between any of the groups (Figure 5.48).

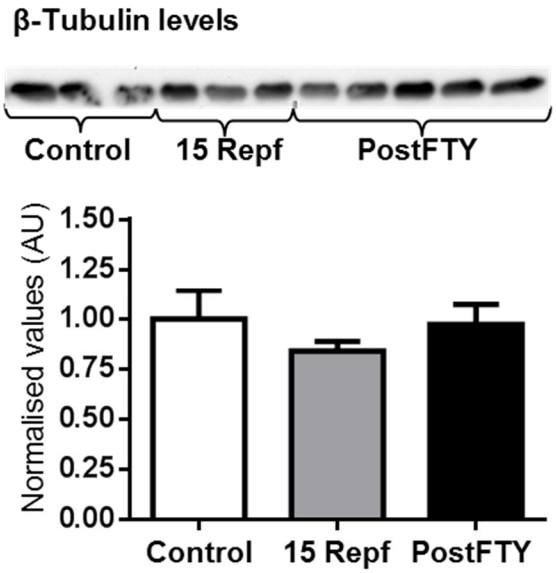


Figure 5.48. Loading control for the effects of reperfusion administered FTY720 at 15 minutes reperfusion. β-Tubulin was used as loading control for the determination of the kinase profiles associated with FTY720 treatment. n=3-5.

Pre- and reperfusion treatment with FTY720: Protein profiles at 35 minutes reperfusion

For these experiments hearts were exposed to 15 minutes of FTY720 (1 μM) administration either directly before 20 minutes GI, or for the first 15 minutes of reperfusion. Hearts were snap frozen after 35 minutes of reperfusion. The control group in these experiments constituted hearts which were also exposed to I/R, but without FTY720 administration.

Protein phosphatase 2A

After 35 minutes of reperfusion FTY720 administered during the first moments of reperfusion was associated with a significant increase in the levels of PP2A-C in comparison with pretreated hearts (Figure 5.49: PreFTY: 0.88±0.03 AU vs. PostFTY: 1.10±0.04 AU, n=5; $P<0.05$). Neither of these groups however differed from control (Control: 1.00±0.09 AU vs. PreFTY: 0.88±0.03 AU vs. PostFTY: 1.10±0.04 AU, n=4-5; NS). The levels of PP2A-C relative to PP2A-A also did not differ between any of the groups. The importance of this elevation in PP2A-C in the PostFTY group is therefore doubtful and probably simply the result of a slight downward fluctuation in PP2A-C in the PreFTY group in combination with a slight upward shift in the PostFTY group.

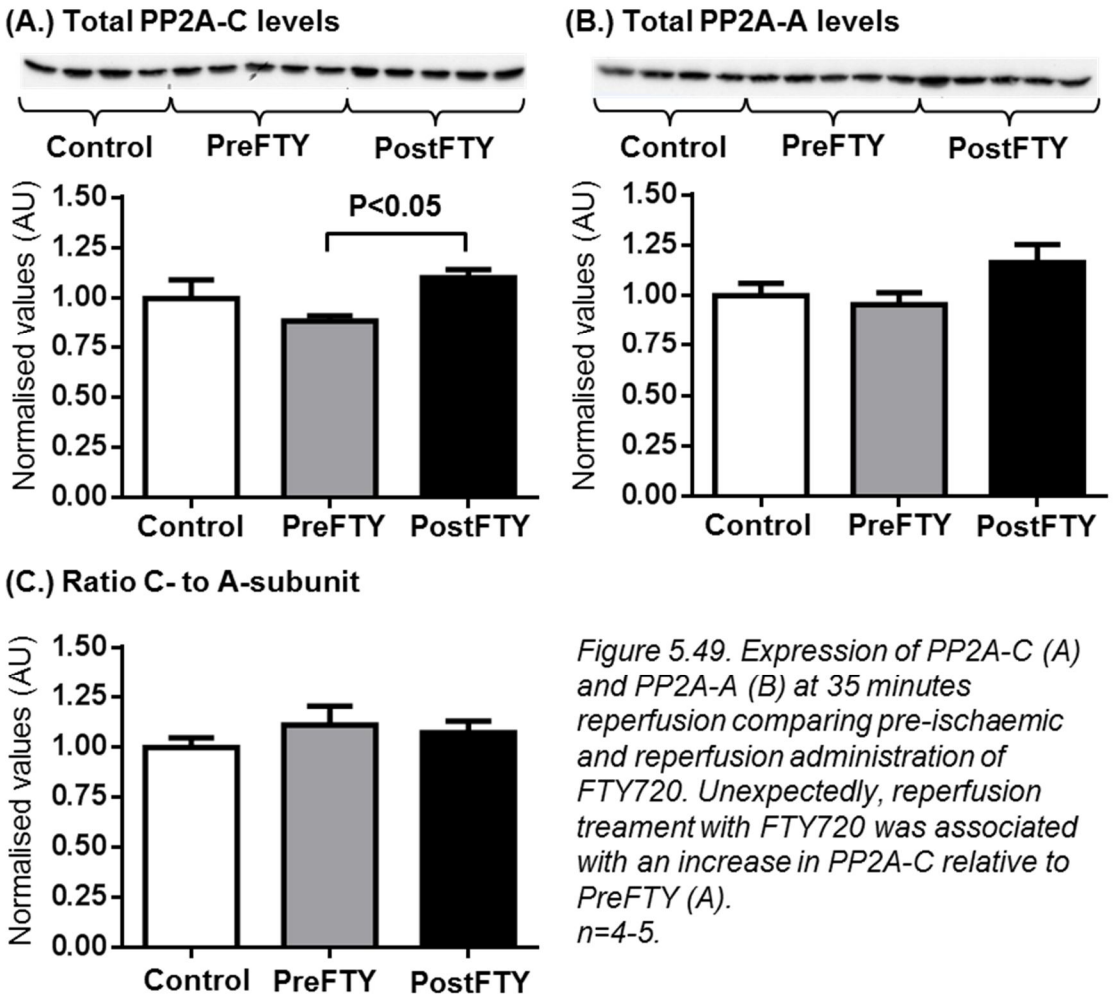


Figure 5.49. Expression of PP2A-C (A) and PP2A-A (B) at 35 minutes reperfusion comparing pre-ischaemic and reperfusion administration of FTY720. Unexpectedly, reperfusion treament with FTY720 was associated with an increase in PP2A-C relative to PreFTY (A).
n=4-5.

Interestingly, FTY720 failed to exert any effect on the phosphorylation of PP2A-C (Figure 5.50) at this time point. This highlights the transient nature of FTY720's effects on PP2A.

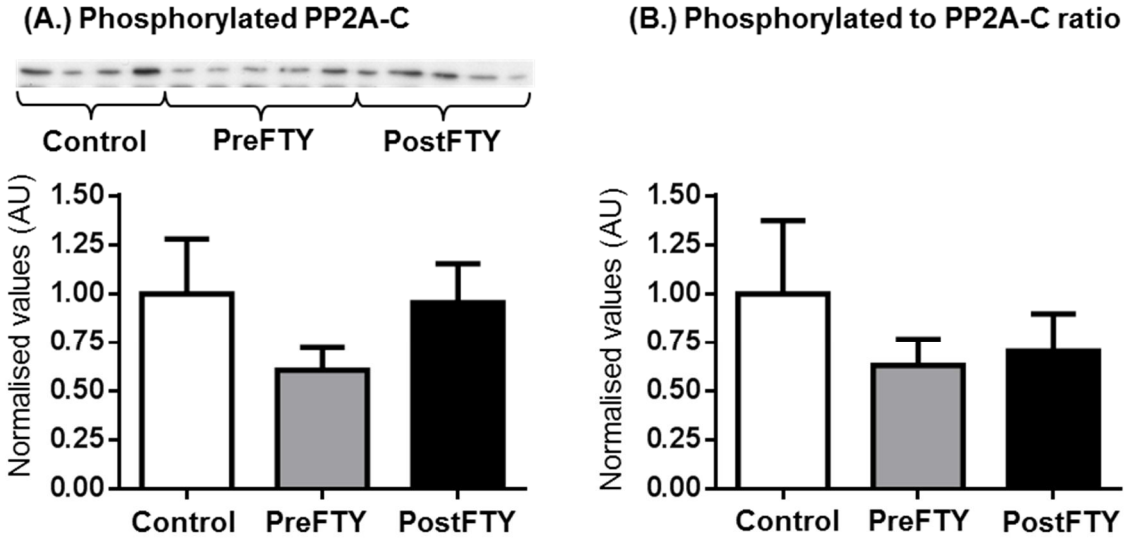


Figure 5.50. Phosphorylation of PP2A-C at 35 minutes reperfusion in hearts treated with FTY720 either prior to 20 minutes GI or at the onset of reperfusion.
n=4-5.

At this stage of the project our stock of antibody against nonmethylated PP2A-C had become quite old and our attempt to probe the blots with it failed. Unfortunately, the supplier from whom we had obtained the antibody had in the meantime decided to take this antibody of the market. We were therefore unable to assess the level of PP2A-C methylation in these final experiments.

Protein kinase B (Akt)

At 35 minutes reperfusion FTY720 exerted no effect on total PKB/Akt levels (Figure 5.51).

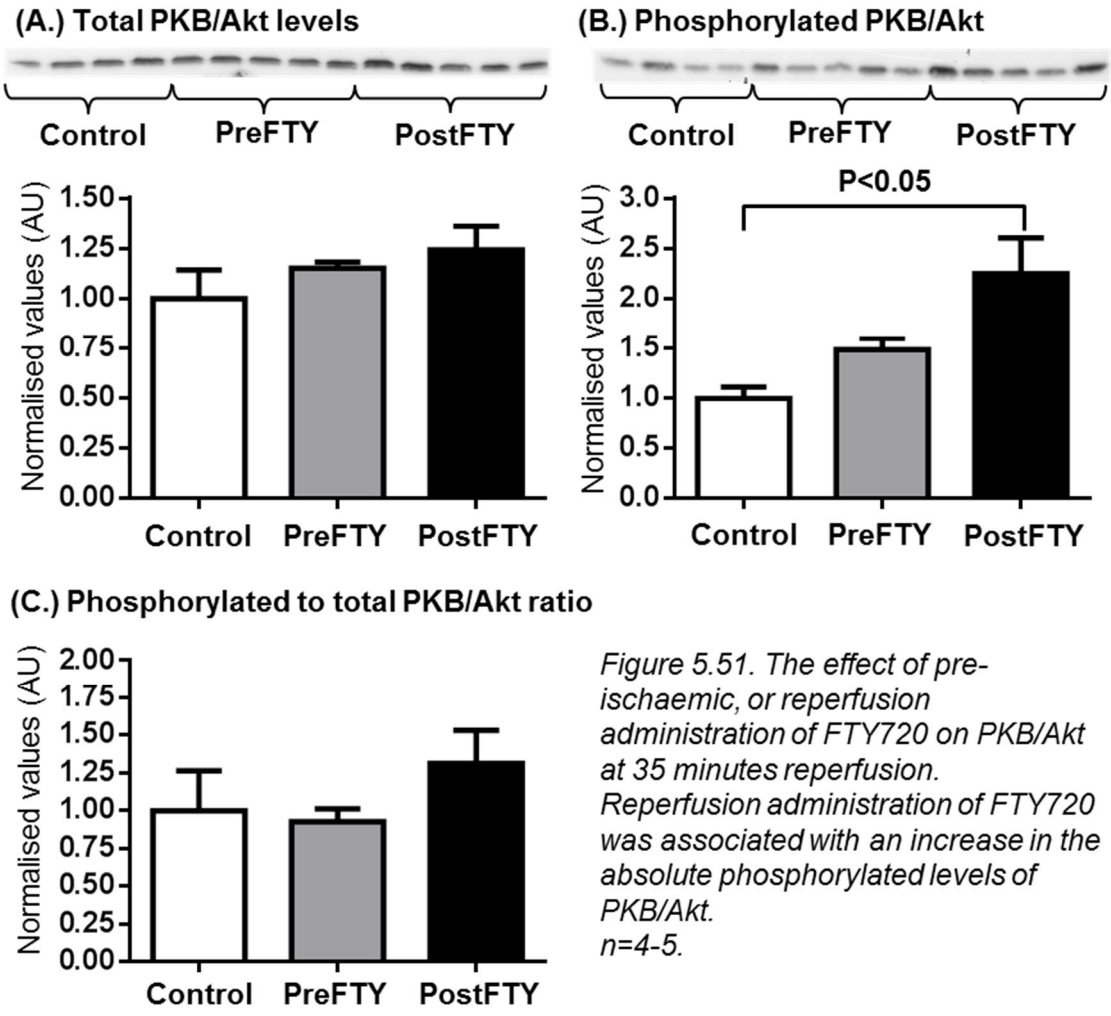


Figure 5.51. The effect of pre-ischaemic, or reperfusion administration of FTY720 on PKB/Akt at 35 minutes reperfusion. Reperfusion administration of FTY720 was associated with an increase in the absolute phosphorylated levels of PKB/Akt. n=4-5.

Reperfusion administration of FTY720 was however associated with a significant increase in the absolute phosphorylated levels of PKB/Akt in comparison with a I/R control (Control: 1.00±0.12 AU vs. PostFTY: 2.25±0.35 AU, n=3-5; P<0.05). Direct comparison of control with PreFTY also revealed a significant difference (Control: 1.00±0.12 AU vs. PreFTY: 1.50±0.11 AU, n=3-5; T-test: P<0.05). Both these differences disappeared when phosphorylation was expressed relative to total PP2A-C (Control: 1.00±0.27 AU vs. PreFTY: 0.93±0.09 AU and PostFTY: 1.31±0.22 AU, n=4-5; NS). The observed increase in phosphorylation can therefore probably be ascribed to residual effects of FTY720, which at this stage of the experiment had diminished considerably, thereby explaining the absence of any significant differences in the phosphorylation of PKBAkt relative to the total pool of PKB/Akt.

Glycogen synthase kinase-3β

Neither pre-ischaemic or reperfusion administration of FTY720 was associated with any changes in the levels of total or phosphorylated GSK-3β (Figure 5.52).

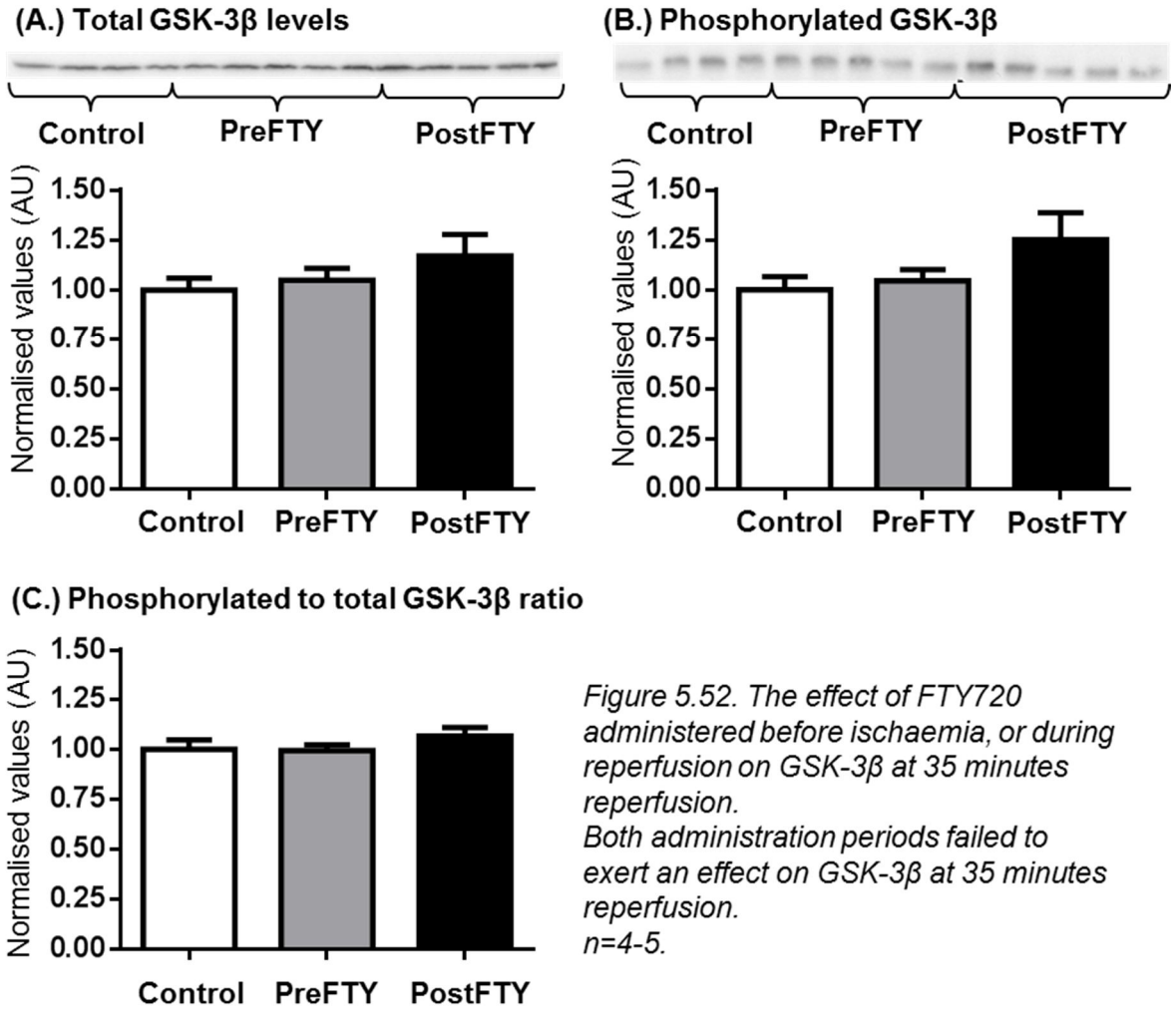


Figure 5.52. The effect of FTY720 administered before ischaemia, or during reperfusion on GSK-3β at 35 minutes reperfusion.
Both administration periods failed to exert an effect on GSK-3β at 35 minutes reperfusion.
n=4-5.

p38 Mitogen activated protein kinase

Similar to PKB/Akt, reperfusion administration of FTY720 was associated with an increase in the absolute phosphorylation of p38 MAPK relative to control (Figure 5.53: Control: 1:00±0.15 AU vs. PostFTY: 2.21±0.26 AU, n=3-5; $P<0.05$). In contrast to the effects of FTY720 on PKB/Akt, its stimulatory effect on p38 MAPK was more robust, with the increase in phosphorylation still evident when expressed relative to total p38 MAPK (Control: 1.00±0.15 AU vs. PostFTY: 2.25±0.26 AU, n=3-5; Dunnett's test: $P<0.05$). PreFTY did not elicit significant effects compared to control.

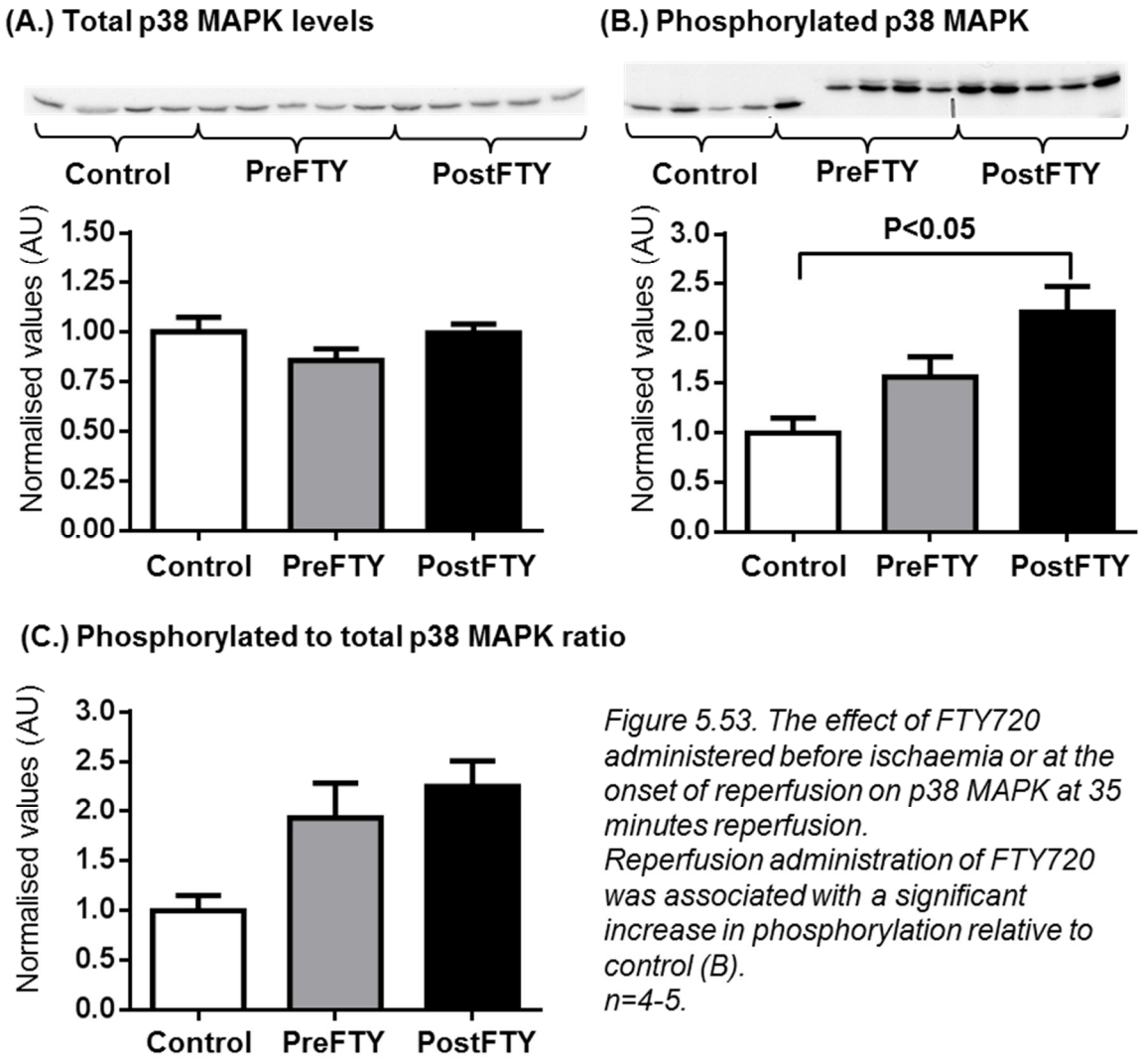


Figure 5.53. The effect of FTY720 administered before ischaemia or at the onset of reperfusion on p38 MAPK at 35 minutes reperfusion. Reperfusion administration of FTY720 was associated with a significant increase in phosphorylation relative to control (B). n=4-5.

Extracellular signal-regulated kinase p42/p44

FTY720 administration, regardless of the time of administration, failed to exert any effects on ERK p42/p44 at 35 minutes reperfusion (Figure 5.54).

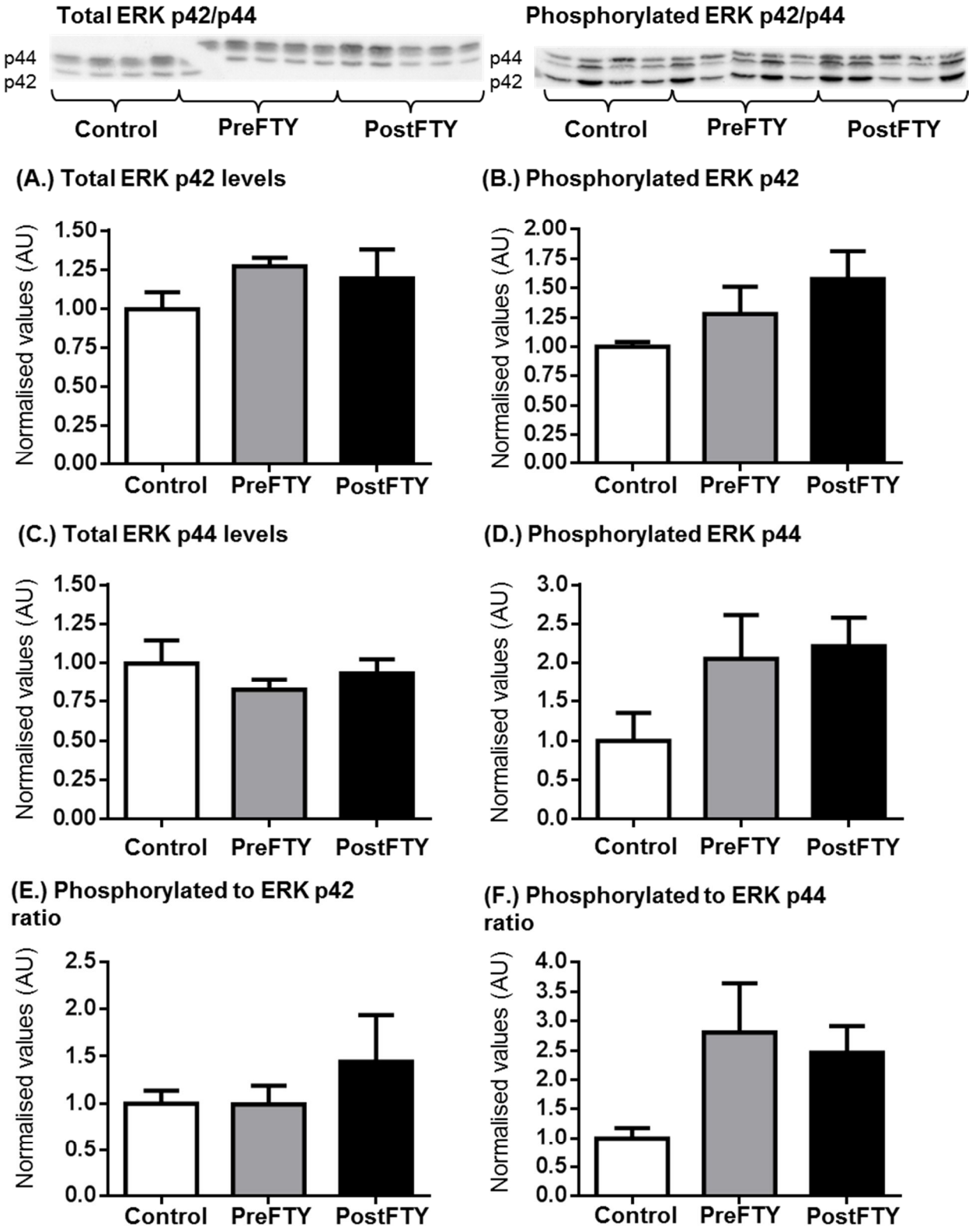


Figure 5.54. ERK p42/p44 at 35 minutes reperfusion following either pre-ischaemic or reperfusion administration of FTY720. FTY720 did not exert any effect on ERK p42/p44.
n=4-5.

Equal loading: β-Tubulin

No significant differences were observed between any of the groups when probed for β-tubulin (Figure 5.55).

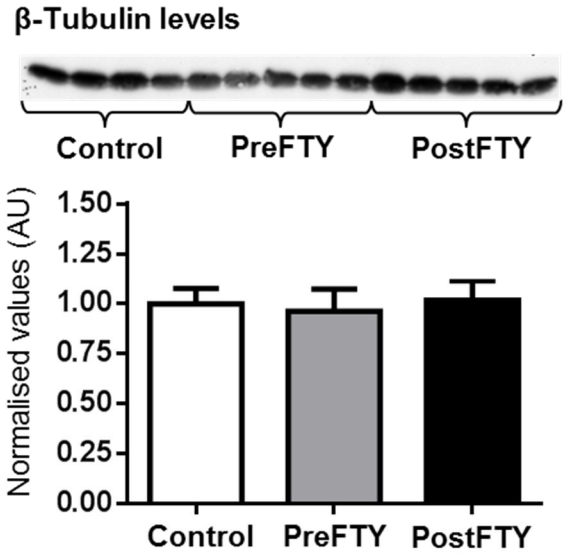


Figure 5.55. Loading control for the effects of pre-ischaemic or reperfusion administration of FTY720, as analyzed at 35 minutes reperfusion. β-Tubulin was used as loading control for the determination of the kinase profiles associated with FTY720 treatment. n=3-5.

Discussion

The results concerning the effects of FTY720 and PP2A activation on the heart are summarized in Figures 5.56-58. In the introduction to this chapter we described the complexity and paradoxical nature of FTY720, characteristics which have become evident in our results. We defined two central aims for this investigation: Our primary aim was to apply FTY720 as a PP2A activator in the setting of myocardial I/R in order to determine the role of PP2A in this setting. In this context, this study is complimentary to Chapter 4 in which we inhibited PP2A. We will therefore also attempt to combine the results and conclusions from these two studies in Chapter 6. A secondary aim was however to simply make a contribution to the body of knowledge concerning the effects of FTY720 on the heart exposed to I/R *per se.* We will first focus on the latter aim and then shift our attention to what our results reveal concerning PP2A.

The effects of FTY720 on the outcomes of myocardial ischaemia / reperfusion

In view of the cardioprotective abilities of S1P (Knapp, 2011; Karliner, 2013), it was expected that FTY720 would also limit myocardial I/R injury – an exciting prospect since FTY720 is already in clinical use as treatment for MS (Chun & Brinkmann, 2011). Previous studies on FTY720 have however failed to deliver an unanimous verdict concerning this (table 5.1). Our results unfortunately also contribute to a deepening of the controversy (Figure 5.56).

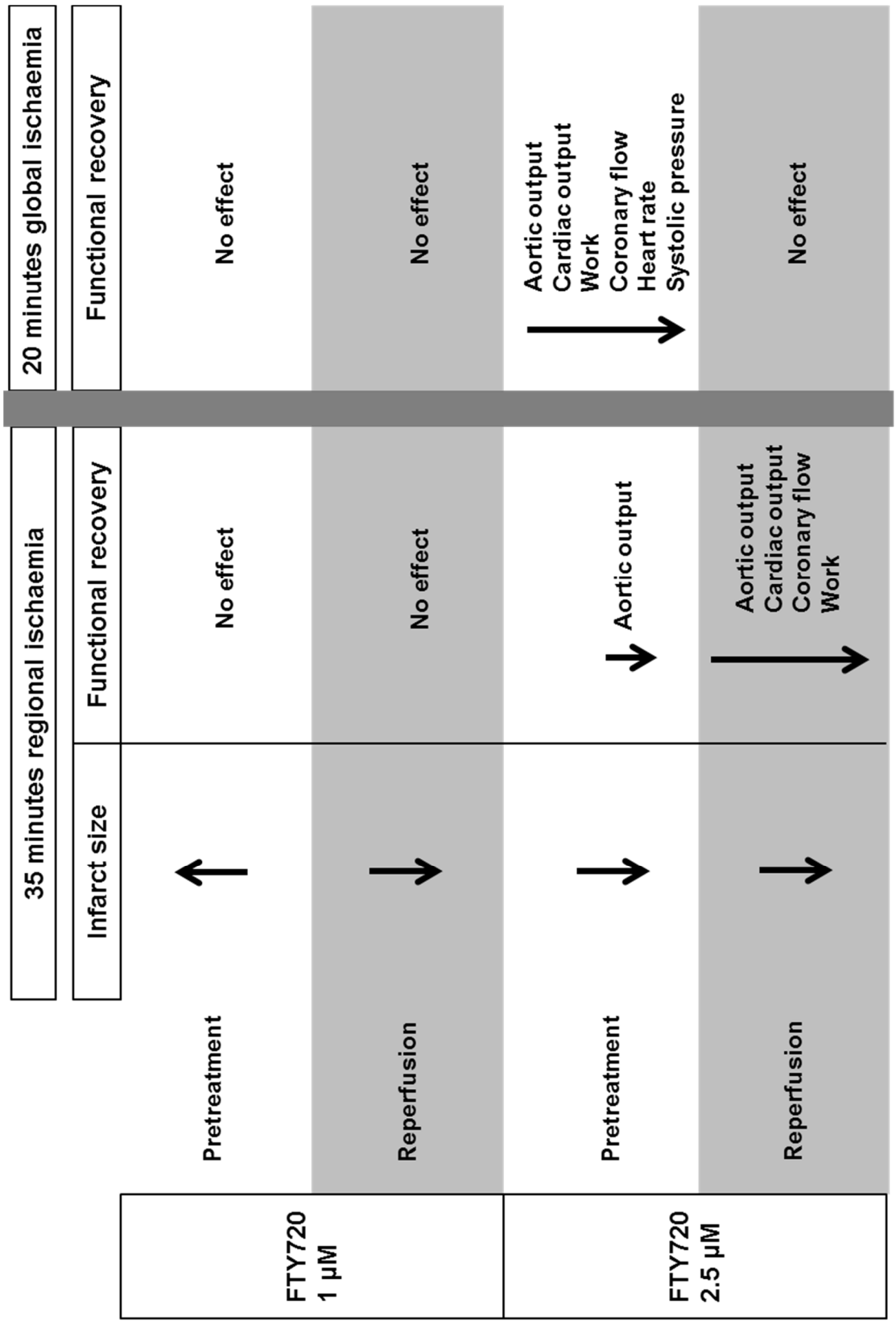


Figure 5.56: The effects of FTY720 perfusion of the isolated rat heart on infarct size and functional recovery following 35 minutes regional ischaemia, as well as functional recovery following 20 minutes global ischaemia.

For these experiments we used two concentrations of FTY720: 1 µM and 2.5 µM. The administration of 1 µM FTY720 during reperfusion limited IFS development, while pretreatment actually augmented IFS (Figure 5.8). Intriguingly, this detrimental effect of FTY720 was alleviated

when a brief period (5 minutes) of reperfusion was allowed between drug administration and index ischaemia (pharmacological preconditioning). None of the administration protocols for 1 µM FTY720 exerted any effect on functional recovery following 35 minutes RI (tables 5.3), except for the preconditioning protocol which was associated with a reduction in CO, probably due to a reduction in heart rate when the heart was reperfused in the work mode (table 5.4). This reduction in heart rate could be explained as a manifestation of the bradycardiac effects of FTY720 (Hla & Brinkmann, 2011).

At 2.5 µM, FTY720 exerted an infarct sparing effect, irrespective of the time of administration (Figure 5.9). This reduction in IFS was however dissociated from functional recovery, indeed functional recovery was compromised in both groups (tables 5.5 and 5.6).

We also administered these doses to hearts exposed to 20 minutes of GI in order to specifically investigate the effects of FTY720 on function. At 1 µM FTY720 exerted no effect on functional recovery (Figure 5.10), while pretreatment with 2.5 µM actually proved detrimental in this regard (Figure 5.11).

These results indicate the following:

- FTY720 exerts dose-specific effects.
- FTY720 exerts different effects on tissue viabillity and functional capacity.
- FTY720 at a concentration of 2.5 µM is detrimental to functional recovery.
- Pretreatment with FTY720 yielded controversial outcomes.

Each of these aspects will be discussed individually.

FTY720 exerts dose-specific effects

This is probably the most obvious observation from our data: pretreatment with 1 µM FTY720 aggravated IFS, while 2.5 µM protected. Also, 1 µM exerted no effects on functional recovery, while with 2.5 µM the reduction in IFS was accompanied by a marked depression in functional recovery. These observations have obvious implications concerning clinical applications of the drug. As already mentioned, Karliner (2009) reported that low doses of sphingosine (of which FTY720 is an analogue) are cardioprotective, while higher dosages are cardiotoxic. Likewise, Vessey *et al.* (2009) found that in old rat hearts 0.4 µM S1P was less effective in limiting IFS following 40 minutes of GI in the isolated heart model, than 0.2 µM (0.2 µM: 27±11% vs. 0.4 µM: 39±6%). It is therefore very plausible that the effects of FTY720 are very dose dependent.

This however raises another question: Are we dealing with FTY720 or P-FTY720? This is a conundrum which was encountered by Oaks and colleagues (2013) and also highlighted by

Vessey *et al.* (2013). This question is important since FTY720 will exert direct effects on intracellular machinery, while P-FTY720 will activate a receptor-mediated signalling cascade. Two observations in our study support the idea that a large percentage of the observed effects is mediated by P-FTY720.

Firstly, FTY720 administration was associated with a potent increase in CF (Figures 5.12 and 5.13). This effect is not surprising since S1P has the ability to stimulate vasodilation in a NOS-dependent manner (Igarashi & Michel, 2009). This effect is however mediated by the occupation and activation of S1P1 and / or S1P3 receptors (Karliner, 2009; Igarashi & Michel, 2009), implying that in our experimental setup FTY720 had become phosphorylated and was eliciting its effects by binding to the S1P receptors. Secondly, preconditioning with FTY720 reduced heart rate during reperfusion, as did pretreatment with 2.5 µM (although all functional parameters were reduced in this protocol). These effects could be due to the bradycardiac effects of S1P (Brinkmann, 2009; Hla & Brinkmann, 2011), and also FTY720 (Schmouder *et al.*, 2012). This is however also dependent on the activation of the S1P1 and S1P3 receptors, again implying that these effects are elicited by P-FTY720.

The phosphorylation of FTY720 is very probable in this setting. It is mediated by SK2, which is the predominant sphingosine kinase in the cytosolic compartment of heart tissue (Vessey *et al.*, 2007; Strub *et al.*, 2010). In addition to this, FTY720 has the ability to activate SK2 whilst inhibiting SK1 (Vessey *et al.*, 2007). The implication of this is that FTY administration itself will progressively favour the phosphorylation of more FTY720. However to what degree the FTY720 which we administered to the hearts became phosphorylated is unknown. It is also possible that a significant portion of the drug remained unphosphorylated, thereby exerting a direct effect on intracellular targets.

FTY720 exerts different effects on tissue viabillity and functional capacity

Excluding pretreatment with 1 µM FTY720, all other administration protocols were associated with a reduction in IFS, however none of the protocols had a beneficial effect on functional recovery, in fact 2.5 µM FTY720 predominantly exerted a negative effect on reperfusion function. The dissociation between IFS and functional recovery data is a well-known phenomenon as was discussed in Chapter 4. As mentioned in the previous chapter, IFS is a measure of tissue death only, while functional recovery is determined by several factors including tissue death (and thus the remaining viable tissue), reversible stunning of tissue and cardiac rhythmicity. The fact that 2.5 µM FTY720 actively contributed to the deterioration of functional capacity indicates that it might not be a simple detachment of function from IFS, but that FTY720 *per se* has harmful effects on function during reperfusion. We will therefore focus on IFS and functional recovery separately.

FT720 and infarct size

Our data indicate that especially the reperfusion-based administration of FTY720 reduces IFS, i.e. increases cell viability, which is in agreement with other studies on the effects of FTY720 (Egom *et al.*, 2011; Vessey *et al.*, 2013), as well as S1P (Vessey *et al.*, 2008; Somers *et al.*, 2012). There is therefore relative concensus regarding the infarct sparing effects of FTY720 when administered at reperfusion. However very low FTY720 concentrations (50 and 500 nM) failed to reduce IFS in an isolated rat heart model exposed to 30 minutes GI, although this was associated with decreased apoptosis (Hofmann *et al.*, 2009).

We also made the surprising observation that pretreatment with 1 µM FTY720 was associated with an increase in IFS (Figure 5.8). This observation is novel, since the effects of pre-ischaemic treatment with FTY720 has received no attention thus far. Preconditioning with S1P has been shown to be cardioprotective (Jin *et al.*, 2002; Vessey *et al*, 2009), however we found FTY720 preconditioning ineffective in influencing IFS. Later in this discussion we will attempt to explain these unexpected results (pages 281 & 292).

FTY720 and functional recovery

Only a few studies focussed on the effects of FTY720 on function alone. We found that 1 µM FTY720 failed to influence functional recovery at all, while 2.5 µM was detrimental to functional recovery.

Our results are in contrast to studies that have reported an increase in functional recovery following regional ischaemia associated with the administration of FTY720 during reperfusion (Hofmann *et al.*, 2009; Vessey *et al.*, 2013). Egom and colleagues (2010) also found that FTY720 reduced rhythmicity disturbances following I/R. The only study that agrees with our results was done in an *in vivo* rat model (Hofmann *et al.*, 2010), which due to systemic effects, cannot be compared to our study.

There are two important differences between our study and the studies reported in the literature which might explain these discrepancies. Firstly, the experimental model used; we used the isolated perfused working heart (see Chapter 2) as opposed to the retrograde perfusion model used by others (Hofmann *et al.*, 2009; Egom *et al.*, 2010; Vessey *et al.*, 2013). This mode of perfusion is energetically more demanding (Taegtmeyer *et* al., 1980) and generates more free radicals (Damerau *et al.,* 1993) compared to simple retrograde perfusion and may therefore compromise functional recovery (Van Vuuren *et al.*, 2008). Secondly, the dose used; we used higher concentrations of FTY720 compared to what was used in the other studies (see table 5.1).

2.5 µM FTY720 is detrimental to functional recovery

We found that 2.5 µM FTY720 reduced functional recovery following 35 minutes of RI, irrespective of whether it was administered before or after sustained ischaemia. Administration of the drug prior to 20 minutes GI also incurred major functional depression at reperfusion.

All other studies concerning FTY720 used concentrations ranging from 0.05 to 0.6 µM (table 5.1). Even the concentrations of S1P used in other studies were very low relative to what we used; ranging from 10 nM (Jin *et al.,* 2002) to 0.4 µM (Vessey *et al.,* 2008 and 2009; Somers *et al.*, 2012). We however tested higher concentrations, since our primary focus was PP2A activation which has been reported to be associated with higher concentrations of FTY720, ranging from 2.5 µM (Neviani *et al.*, 2007) to 10 µM (Saddoughi *et al.*, 2013). Although it could be argued that high doses of FTY720 exert toxic effects, this seems unlikely since the initial work on FTY720 showed that it did not elicit any visible toxic effects, even at high doses (Neviani *et al.*, 2007; Oaks *et al.*, 2013). Although Karliner (2009) noted potential cardiotoxic effects of sphingosine at 5 µM, it is a dose twice as high as our highest dose. Challenging these high levels, Theilmeier *et al*. (2006) even reported a cardioprotective effect with 10 µM S1P in a rat neonatal cardiomyocyte model.

Alternatively our results simply reveal the ability of FTY720 and P-FTY720 to exert differential effects on different components of cardiovascular function during I/R, thereby limiting cell death while exerting detrimental effects on function.

Pretreatment with FTY720 yielded controversial results

As mentioned before, to our knowledge there are no studies which have investigated the effects of FTY720 administered prior to ischaemia on the development of I/R injury. Our results concerning this is novel, albeit not promising. Pretreatment with 1 µM FTY720 increased IFS, while pretreatment with 2.5 µM reduced functional recovery following both 35 minutes regional ischaemia, as well as 20 minutes global ischaemia.

The potential mechanisms involved will be discussed in the next section.

Mechanisms explaining the effects of FTY720

This study is limited to only describing the effects of 1 and 2.5 µM FTY720 on the outcomes associated with myocardial I/R. We can therefore only speculate regarding the mechanisms involved within this setting. We will focus our explanation on the two aspects of FTY720 as indicated by our results: namely its effects on cardioprotection, and the possible negative effects on myocardial I/R injury.

FTY720 in cardioprotection

The cardioprotective effects observed in conjunction with FTY720 administration pertains solely to its ability to limit the development of IFS following I/R. We suggest three possible mechanisms by which FTY720 can elicit an infarct sparing effect:

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1.) Increasing the amount of S1P in the heart.

The amount of S1P in the heart is a function of the balance between synthesis and degradation. Two important enzymes in this regard are sphingosine kinase (SK, both isoforms 1 and 2), which phosphorylates sphingosine to S1P; and S1P lyase (SPL), which is involved in the irreversibe degradation of S1P (Knapp, 2011). Both of these enzymes have been shown to be important in the context of cardioprotection.

Sustained ischaemia is associated with a reduction in the activity of SK, which does not recover rapidly during reperfusion (Karliner, 2009). This loss in SK activity is closely linked to myocardial function and has been shown to be a target of IPC mediated cardioprotection, with IPC limiting ischaemic loss of SK activity and stimulating a rapid recovery of SK function during reperfusion (Vessey *et al.*, 2006). Combined with this, animal knockout models of SK2 have been found to be more sensitive to I/R injury than control hearts and are resistant to IPC-induced cardioprotection (Vessey *et al.*, 2011; Gomez *et al.*, 2011). These data identify SK2 as a pro-survival kinase. Much less is known regarding SPL in the setting of myocardial I/R. In 2011 Bandhuvula and colleagues reported that sustained ischaemia was associated with an increase in the activity of SPL. Inhibition or knockout of SPL was associated with a reduction in IFS and an improvement in post-ischaemic function.

FTY720 has been shown to modulate both these enzymes. Although FTY720 has been shown to inhibit SK1, it has the opposite effect on SK2 (Vessey *et al.*, 2007). FTY720 has also been implicated in the inhibition of SPL (Bandhuvula *et al.*, 2005; KleinJan *et al.*, 2013; Reina *et al.*, 2013). This suggests that FTY720 can potentially inhibit the detrimental SPL and activate the cardioprotective SK2. This will eventually favour an increase in S1P levels, which is the mechanism which has been proposed to explain the influence of these enzymes on I/R injury (Vessey *et al.*, 2006 and 2011; Bandhuvula *et al.*, 2011). An elevation in S1P levels then induces a cardioprotective effect by binding to and activating the S1P receptors. In this regard it is noteworthy that Bandhuvula and colleagues (2005) reported that systemic administration of FTY720 to a mouse model led to a transient increase in S1P levels in the thymus and spleen.

2.) Pro-survival signalling through the S1P signalling pathways.

As discussed above, FTY720 can theoretically induce an increase in S1P levels, initiating cardioprotective signalling. It must however also be kept in mind that once phosphorylated, P-FTY720 can also bind to and activate S1P receptors, thereby also contributing to the activation of these pro-survival pathways. In this regard S1P1 and S1P3 receptors have been implicated in S1P protection (Theilmeier *et al.*, 2006; Knapp, 2011). Following the activation of these GCPR's, PKB/Akt and Stat3 are recruited into the pathway, which eventually culminates in protection (Vessey *et al.*, 2008; Knapp, 2012; Somers *et al.*, 2012).

3.) Direct effects on signalling (S1P independent signalling).

In 2008 Vessey and colleagues reported that sphingosine-induced protection is mediated through other pathways than S1P protection, namely protein kinase G (PKG) and PKA activation. Interestingly, sphingosine and S1P both eventually signal through PKB/Akt. A couple of years later the same group reported similiar results for FTY720 (Vessey *et al.*, 2013). They found that postconditioning with FTY720 conferred protection independently of S1P signalling but, similiar to sphingosine, it is dependent on PKG and PKA activity. These results demonstrate that FTY720 can induce protection separately from S1P signalling.

FTY720 therefore has several potential pathways and mechanisms by which it can elicit a protective effect. An explanation for its detrimental effects is however far less forthcoming.

FT720 augmenting I/R injury

We found that pretreatment with 1 µM FTY720 increased IFS, an effect which was avoided when FTY720 administration was followed by a brief period of reperfusion. Combined with the negative effects on functional recovery which were elicited by 2.5 µM FTY720 (Figure 5.56), these observations reveal a harmful side to FTY720 which can, at least partially, be explained as follows.

As discussed previously, high concentrations of S1P can exert cardiotoxic effects. It can therefore not be ruled out that the concentrations of FTY720 which we used may have been high enough to exert similar cardiotoxic effects. This theory is particularly attractive when taking into account the fact that at 2.5 µM, and not 1 µM, FTY720 elicited the most detrimental effects on function. However, the dosages used were not that high (see Karliner, 2009; Theilmeier *et al.*, 2006) and FTY720 has been reported to exert no toxic effects, even after prolonged exposure to relatively high concentrations (Neviani *et al.*, 2007; Oaks *et al.*, 2013). In addition, it should be kept in mind that the harmful effects of FTY20 (2.5 µM) on function become evident during reperfusion, suggesting that the drug exerts its harmful effects on ischaemic damaged tissue.

Alternatively, in view of the reduction in infarct size seen in all instances (except pretreatment and preconditioning with 1 µM), it might simply be that the functional suppression is due to the negative inotropic and bradycardiac effects associated with S1P1 and S1P3 activation (Means & Brown, 2009; Hla & Brinkmann, 2011). This possibility is corroborated by the fact that the reduction in CO during reperfusion associated with FTY720 preconditioning occurred in conjunction with a reduction in heart rate, while the functional depression seen in conjunction with 2.5 µM FTY720 was also associated with a reduction in total work (reperfusion administration following 35 minutes RI), as well as a reduction in work, systolic pressure and heart rate (administration prior to 20 minutes GI).

FTY720 and coronary flow

A prominent feature of S1P is its ability to either induce vasodilation or vasoconstriction, almost paradoxically, in both instances by inducing an increase in intracellular Ca^{2+} levels, but in different tissues. In the case of vasodilation, increased endothelial cellular Ca^{2+} contributes to NOS activation, and generation of the vasodilator NO. In vascular smooth muscle, it however mediates contraction leading to constriction (Igarashi & Michel, 2009). We observed that FTY720 exerted both vasodilatory- and constrictive effects in our model. On the one hand, FTY720 administration itself was associated with an outspoken increase in CF, irrespective whether it was 1 or 2.5 µM FTY720 being administered, either before ischaemia (Figure 5.12 A) or at the onset of reperfusion (Figure 5.13 A&B). Unexpectedly, exposure of the hearts to I/R in conjunction with FTY720 (as in the pre-ischaemic treatment groups) reversed this effect, so that FTY720 exerted a transient vasconstrictive effect during the first minutes of reperfusion (Figure 5.12 B). The mechanism behind this is unknown. Possibly I/R adds to a FTY720-mediated increase in intracellular Ca^{2+} levels in the vascular smooth muscle, which then dominates the NO-mediated vasodilatory effects of FTY720 – especially if these latter effects which were present before ischaemia are fading during ischaemia and reperfusion.

These effects of FTY720 on CF are so outspoken that it is tempting to directly link them to either the infarct sparing, or function depressing effects of FTY720. Keeping in mind the dose dependent effects of FTY720 on functional recovery and IFS (Figure 5.56) and comparing these outcomes to the homogenous pattern of CF effects exerted by both concentrations of FTY720 (Figure 5.13 C), it becomes evident that there is no clear pattern linking changes in CF, either as a causal mechanism or a consequence, to the outcomes exerted by FTY720.

FTY720 in myocardial I/R injury: summary

In our model FTY720 administration at both 1 and 2.5 µM was associated with a reduction in infarct size following 35 minutes of RI, except for the pre-ischaemic and preconditioning administration of 1 µM FTY720. This cardioprotective effect of FTY720 can be ascribed to the fact

that it is a sphingosine analogue which contributes to the activation of the cardioprotective sphingosine and S1P pathways. These infarct sparing effects however did not extend to functional recovery. In fact 2.5 μM FTY720 almost consistently reduced functional ability during reperfusion. This could be due to the bradycardiac and negative inotropic effects of FTY720. FTY720 also exerted profound effects on CF, although the importance of this in relation to the outcomes associated with FTY720 treatment is unknown. Our data indicate that FTY720 maintains tissue viability, while paradoxically, undermining functional capability.

FTY720 as an activator of PP2A in the setting of myocardial I/R injury

The primary motivation behind our investigation into the effects of FTY720 was the fact that FTY720 has been associated with the activation of PP2A, both directly through interaction with the PP2A inhibitor SET (Oaks *et al.*, 2013; Saddoughi *et al*., 2013), as well as through Pak1 signalling (Ke *et al.*, 2012). For this aspect of the study we specifically used 1 μM FTY720, since it represented a relatively low dose of FTY720 with the ability to activate PP2A (Figure 5.7). We will therefore now focus our attention on the effects of FTY720 as a PP2A activator on the signalling components involved in RISK (PKB/Akt – GSK-3β, and ERK p42/p44) and p38 MAPK signalling. From there we will revisit our data concerning the effects of 1 μM FTY720 on IFS and functional recovery in order to ascertain if some of these effects can be ascribed to PP2A activity.

The effects of FTY720 mediated PP2A activation on pro-survival and MAPK signalling

In these experiments FTY720 was administered either directly prior to sustained ischaemia or at the onset of reperfusion. Hearts were then collected at different time points during the perfusion protocol and protein profiles determined using standard Western blotting techniques. An overview of the results are shown in Figures 5.57 and 5.58. We will discuss the effects of FTY720 on each signalling component individually.

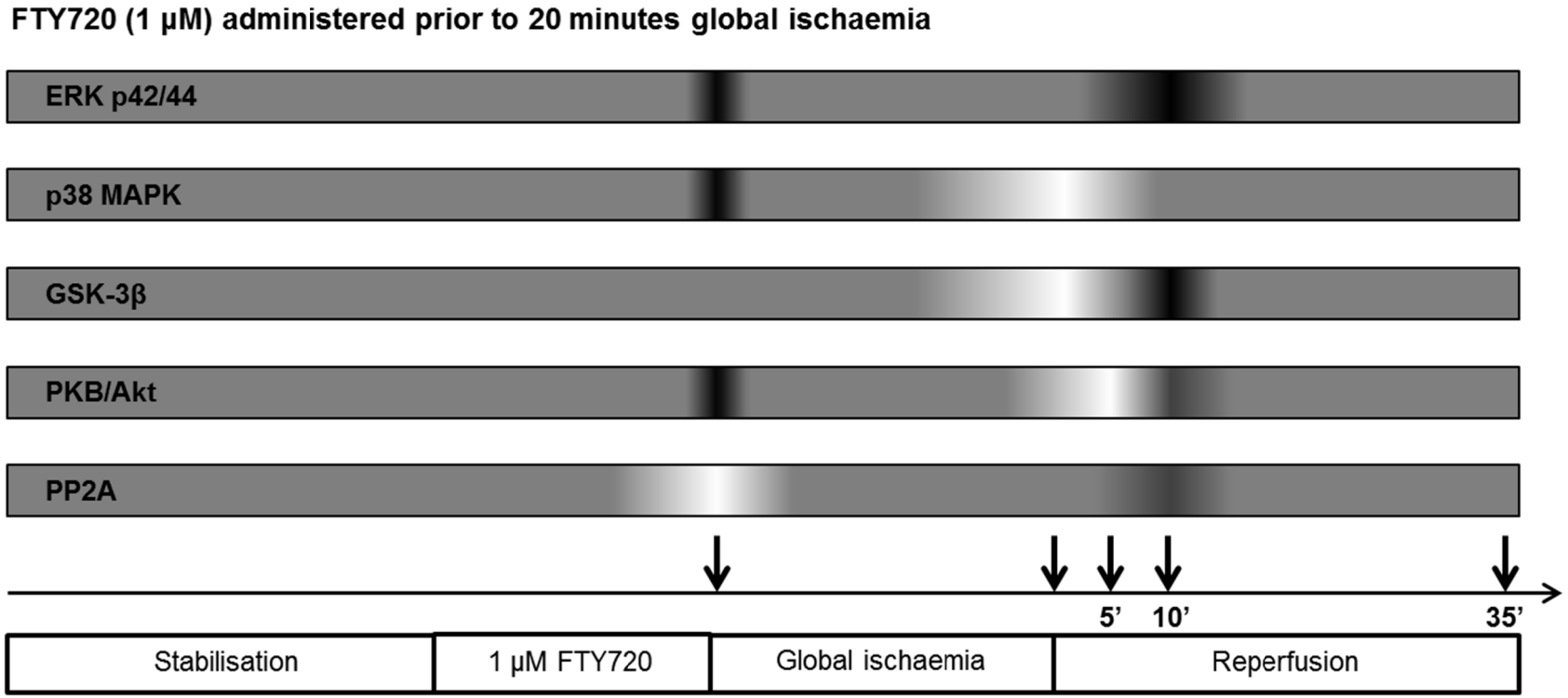


Figure 5.57. Summary of the effects of FTY720 (1 µM) administered prior to 20 minutes GI on the phosphorylation of PP2A, PKB/Akt, GSK-3β, p38 MAPK and ERK p42/p44. This figure is an attempt to illustrate the patterns of phosphorylation associated with FTY720 treatment. For the actual data please see the relevant sections and figures in the text.
Legend:

Potent increase in phosphorylation (FTY720 vs. I/R control)

Less pronounced increase in phosphorylation (FTY720 vs. non-ischaemic control)

Reduction in phosphorylation (FTY720 vs. I/R control)

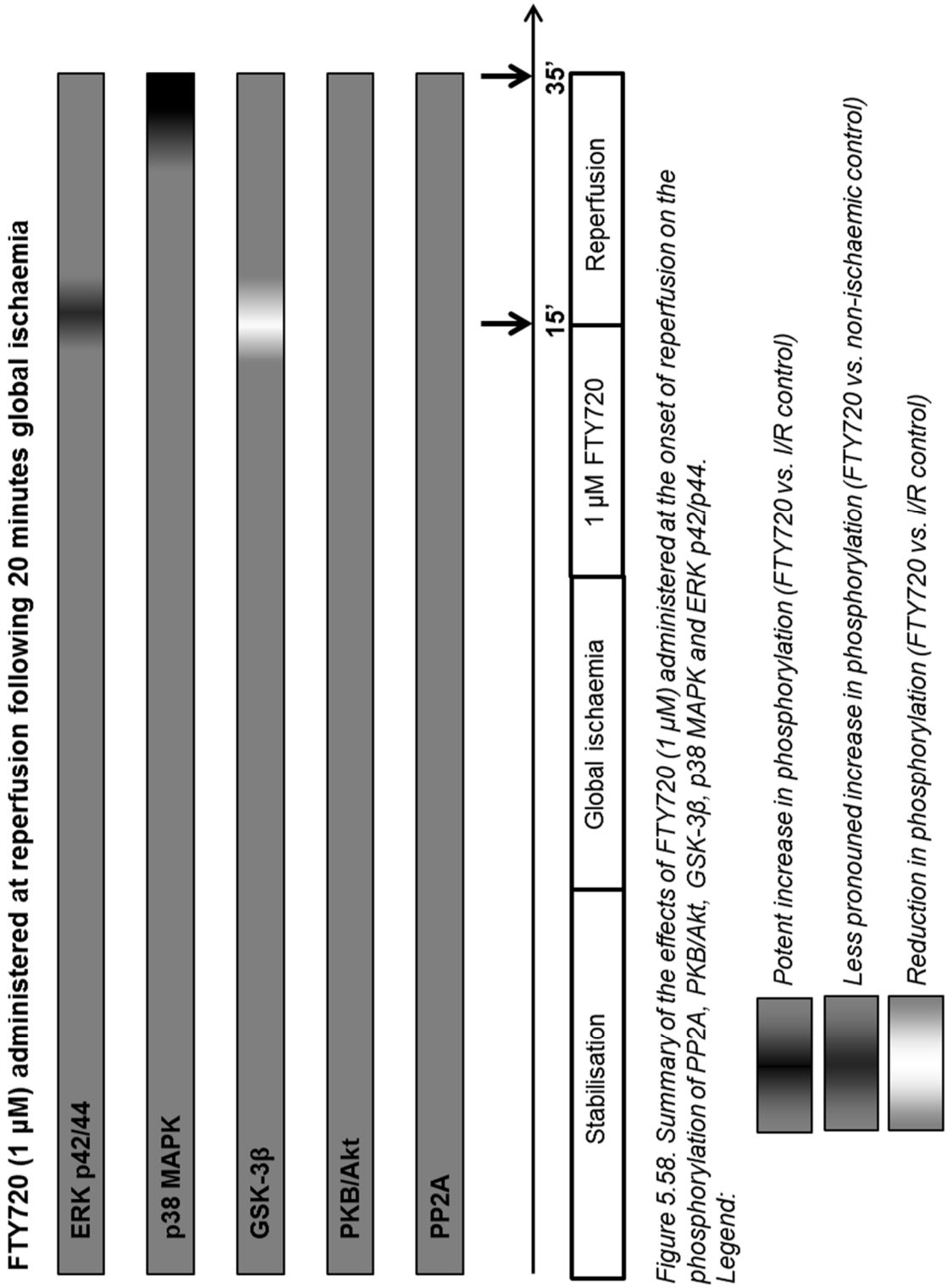


Figure 5.58. Summary of the effects of FTY720 (1 µM) administered at the onset of reperfusion on the phosphorylation of PP2A, PKB/Akt, GSK-3β, p38 MAPK and ERK p42/p44.
Legend:

Protein phosphatase 2A

As expected FTY720 administration prior to ischaemia was associated with a reduction in the phosphorylation of PP2A (Figure 5.15), which is indicative of an increase in PP2A activity. These data agree with our initial investigations into the effects of 1 µM FTY720 on PP2A (Figure 5.7). It is therefore surprising that reperfusion administration of FTY720 failed to elicit any effects on PP2A phosphorylation (Figure 5.43). It was however associated with a reduction in methylation – indicating a possible change in holo-enzyme assembly, although the ratio of PP2A-C to PP2A-A

remained unchanged. It therefore seems as if the effect of I/R *per se* to inhibit PP2A by increasing its phosphorylation (see Chapter 3) dominated the activation effects of FTY720. It is therefore uncertain to what degree FTY720 administered at reperfusion could activate PP2A.

The effect of FTY720 on PP2A phosphorylation was also very transient. Although pretreatment reduced PP2A phosphorylation directly before ischaemia, this was no longer visible at 20 minutes GI where the standard increase in both phosphorylation and nonmethylation of PP2A (Figure 5.22) was observed, as previously reported in Chapter 3. These data indicate that the effects of I/R *per se* on PP2A are quite robust and overshadow the potential effects of FTY720 on PP2A. It could however also mean that the signalling effects of FTY720 are intrinsically short-lived.

We therefore have evidence that PP2A was activated by FTY720 administration at the onset of ischaemia, with no indication that this also continued during ischaemia and reperfusion. That being said, it should be remembered that PP2A activity is much more complex than its phosphorylation or lack thereof.

Protein kinase B/Akt

Administration of FTY720 at baseline (prior to ischaemia) elicited a pronounced increase in the phosphorylation of PKB/Akt (Figure 5.16). This activation was however less outspoken when FTY720 was administered at reperfusion (Figure 5.44), possibly because PKB/Akt was already phosphorylated to near maximum levels due to reperfusion *per se.* At 35 minutes reperfusion there was less pronounced PKB/Akt activation in the PostFTY group (Figure 5.51), possibly a consequence of FTY720 administration, which is diminishing as reperfusion progressed. The activation of PKB/Akt due to FTY720 administration has been reported by several other studies and is indeed postulated to be one of the cornerstones of the cardioprotective effects which have been ascribed to FTY720 (Hofmann *et al.*, 2009; Egom *et al.*, 2010 and 2011). This is also not surprising, since PKB/Akt activation is also stimulated by both sphingosine and S1P signalling (Vessey *et al*., 2008; Somers *et al.*, 2012).

This activation of PKB/Akt is suppressed during ischaemia (a well established phenomenon), remaining reduced at 5 minutes reperfusion relative to reperfusion alone, although it is still elevated relative to non-I/R control (Figure 5.30). This is a very outspoken effect, which wanes within the next 5 minutes so that at 10 minutes reperfusion PKB/Akt phosphorylation is elevated in the PreFTY group relative to a non-I/R control (Figure 5.37), an effect which had disappeared by 35 minutes reperfusion (Figure 5.51). These data indicate that PP2A activity might be increased at the onset of reperfusion in the PreFTY group which might be responsible for the reduction in PKB/Akt phosphorylation at 5 minutes reperfusion. These PP2A-mediated effects are however transient and rapidly replaced by the stimulatory effects of FTY720. If this interpretation of the data

is correct, it also implies that FTY720 administration is simultaneously activating several very different pathways, which are possibly even opposing each other.

Glycogen synthase kinase 3-β

Pre-ischaemic administration of FTY720 did not result in an increase in the phosphorylation of GSK-3β (Figure 5.17) during stabilisation and remained unchanged until the end of 20 min global ischaemia. However, the ischaemia alone group presented with an elevation in phosphorylation relative to non-I/R control (Figure 5.24), indicative of a suppression of GSK-3β activity (since phosphorylation of GSK-3β is associated with a reduction in its activity). This is a strong indication that FTY720 pretreatment favoured the dephosphorylation (and therefore activation) of GSK-3β during ischaemia. This effect however diminished during reperfusion, so that by 5 minutes reperfusion both PreFTY and reperfusion alone presented with increased phosphorylation relative to non-I/R control (Figure 5.31). Similar to PKB/Akt, the phosphorylation of GSK-3β actually increased in the PreFTY group during reperfusion, to such an extent that it reached statistical significance at 10 minutes reperfusion (Figure 5.38). When FTY720 was administered during reperfusion it showed a very strong trend for reducing the degree of reperfusion-induced GSK-3β phosphorylation (Figure 5.45). At 35 minutes reperfusion no differences are associated with any of the FTY720 treatment regimes.

There are four important conclusions which can be made from this data. Firstly, as mentioned in Chapter 4, it should be kept in mind that the effects of phosphatase activity modulation is best seen on a background of kinase-mediated activation. This explains the lack of an effect of FTY720 in GSK-3β prior to ischaemia, in contrast to the effect at the end of ischaemia. Secondly, the effects of FTY720 concerning the dephosphorylation of GSK-3β at the end of ischaemia is probably PP2A-mediated, thereby identifying GSK-3β as a potential substrate for PP2A during ischaemia. Thirdly, as was seen with PKB/Akt activation, these dephosphorylation effects of FTY720 are soon replaced with increased phosphorylation relative to I/R control, thereby indicating the co-activation of stimulatory pathways alongside the increase in PP2A activity. Fourthly, the PP2A mediated effects of FTY720 are very transient. This could be due to the intrinsic nature of the interaction between FTY720 and PP2A, or it could simply be due to the intracellular milieu being dominated by I/R associated pathways.

p38 Mitogen activated protein kinase

As was the case for both PKB and PP2A, baseline administration of FTY720 induced an increase in p38 MAPK activity, as indicated by an increase in its phosphorylation (Figure 5.18). However at the end of sustained ischaemia, p38 MAPK phosphorylation resembled that of GSK-3β, with a reduction in phosphorylation at 20 minutes GI relative to the ischaemic control (Figure 5.25). It must however be noted that both ischaemia alone, as well as PreFTY presented with a

substantially higher degree of phosphorylation than the normal perfusion control. The FTY720 mediated suppression of p38 phosphorylation however rapidly dissipated during early reperfusion so that both the I/R and PreFTY groups presented with a similiar degree of elevation in phosphorylation relative to the non-I/R control (Figures 5.32 and 5.39).

Unexpectedly, given the effects of FTY720 prior to ischaemia, administration of FTY720 at reperfusion failed to significantly elevate p38 MAPK phosphorylation (Figure 5.46). This is however similar to what we observed with PKB/Akt, and can possibly be ascribed to the already elevated phosphorylation of p38 MAPK due to reperfusion. Reperfusion treatment with FTY720 was however associated with a slight increase in the phosphorylation of p38 MAPK at 35 minutes reperfusion (Figure 5.53); possibly a remnant of a more potent effect of FTY720 on p38 phosphorylation earlier in reperfusion which might be more evident at a time point between 15 and 35 minutes reperfusion.

We therefore here again observe the phenomenon that FTY720 activates multiple pathways, some inducing an increase in p38 MAPK phosphorylation, while others oppose it. The complicating factor with p38 MAPK is the fact that it might be a link between these two pathways, since p38 MAPK activation has been shown to be upstream of PP2A activation (see introduction to Chapter 4).

Extracellular signal-regulated kinase p42/p44

Fitting in with the established trend, ERK p42/p44 was phosphorylated by the administration of FTY720 under baseline conditions (Figure 5.19). At 20 minutes GI the phosphorylation of ERK p42/p44 was equally supressed in both the ischaemic, as well as PreFTY groups, relative to the non-I/R control (Figure 5.26). Deviating from the other kinases, FTY720 showed no suppressive effect on the phosphorylation of ERK p42/p44, in fact quite the opposite. At both 5 and 10 minutes reperfusion PreFTY presented with elevated phosphorylation of ERK p42/p44 (Figures 5.33 and 5.40). In line with these results, reperfusion treatment with FTY720 was also associated with an increase in ERK p42/p44 phosphorylation, although not as robust since it only involved ERK p44 and was only evident in comparison to non-I/R controls (Figure 5.47). At 35 minutes reperfusion, the effects of both Pre- and PostFTY720 treatment on ERK p42/p44 phosphorylation had diminished and was no longer evident.

ERK p42/p44 therefore followed a different pattern than the other kinases in the sense that FTY720 seemed to only activate it, with no hint of a co-activated dephosphorylation pathway. These results therefore imply that PP2A is not an important phosphatase of ERK p42/p44 in the context of myocardial I/R injury. It is noteworthy that our results differ from those published by Hofmann and colleagues (2009) who reported that FTY720 exerted no effect on ERK p42/p44 phosphorylation in their experimental setup. Their experimental conditions differed from ours in

several ways: FTY720 was administered at half the dose we used (500 nM) and they exposed their isolated rat hearts to 30 minutes GI and 90 minutes reperfusion. It was also only at 90 minutes reperfusion that they collected tissue for protein analysis. Their isolated heart experiments also did not include work mode perfusion. These differences with regards to concentration administered, as well as stress that the hearts were exposed to and reperfusion time, make it very difficult to compare results. Interestingly they also reported that FTY720 activated PKB/Akt, similar to our results, thereby indicating the robustness of this particular effect. Contributing to the complexity, they also found that 1 µM could in fact increase ERK p42/p44 phosphorylation (but not PKB/Akt) in human right atrial muscle strips when exposed to 90 minutes SI followed by 120 minutes reperfusion.

Summary and conclusion

Of the two administration protocols tested, the pre-ischaemic application of FTY720 gave the most information. A graphic summary of the effects of FTY720 in these experiments is given in Figure 5.59.

The interpretation of the data is challenging, since FTY720 is not the only signalling modifier involved in our experiments, I/R *per se* also elicits a signalling mediated response, which according to our data seemed to compete with and oppose some of the effects elicited by FTY720. It must also be kept in mind that PP2A activity is not only determined by its phosphorylation state. Its cellular location and substrate specificity are also very dynamic and also influenced by I/R (see Chapter 3). The implication of this is that our results might be very specific for I/R, and caution must be taken when attempting to extrapolate our results and conclusions to other settings.

All of that being said, the following picture seems to emerge (Figure 5.60). FTY720 activates several pathways simultaneously, probably though direct effects as well as S1P receptor mediated activation. This culminates in the immediate activation of ERK p42/p44, PKB/Akt, p38 MAPK and PP2A (as measured prior to ischaemia). At the end of sustained GI and the onset of reperfusion PP2A dominates the signalling pathways and dephosphorylates PKB/Akt, GSK-3β and p38 MAPK. This could either reflect the temporal effects of FTY720 on PP2A signalling, or it could be due to the fact that some FTY720 is still present in the heart at the end of ischaemia and onset of reperfusion. For GSK-3β this requires some form of pre-phosphorylation, induced by sustained ischaemia in our experimental setup. It is theoretically also possible that PKB/Akt could phosphorylate GSK-3β under FTY720 stimulation, although it is very unlikely given that ischaemia is associated with the dephosphorylation and inactivation of PKB/Akt.

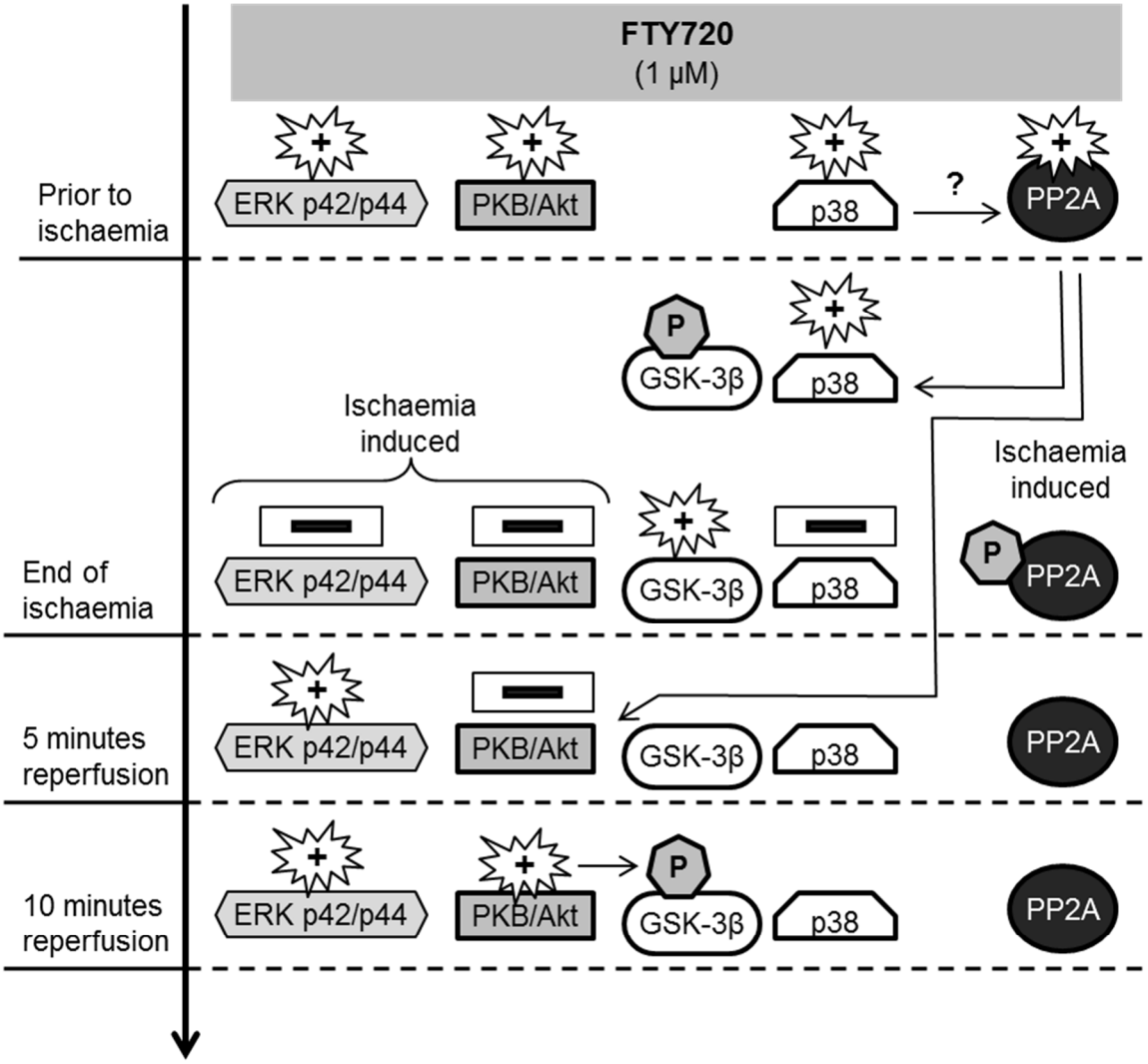


Figure 5.59: The effects of FTY720 administered prior to sustained ischaemia on the profiles of PP2A, PKB/Akt, GSK-3β, p38 MAPK and ERK p42/p44.
This figure is not meant to be a precise description of the effects of FTY720, but rather to give an overview of the effects of FTY720 on these proteins. Where possible, we omitted the effects of I/R on its own on these kinases.
Legend:

Activation

Inhibition

Phosphorylation; this was included specifically for GSK-3β and PP2A in order to avoid confusion, since they are the only enzymes in the figure which are deactivated by phosphorylation.

As discussed in the introduction to this chapter, FTY720 can potentially activate PP2A either directly, or via the activation of signalling pathways. In this regard Pak-1 has been identified as a potential upstream activator of PP2A (Ke *et al.*, 2012). Our results however also show that FTY720 can activate p38 MAPK, which has also been implicated as a potential activator of PP2A (see the introduction to Chapter 4). Irrespective of the mechanism how PP2A is activated by FTY720, it is a very transient phenomenon. As soon as PP2A activity dissipates, the activation of PKB/Akt and p38 MAPK dominate again (5 and 10 minutes reperfusion, as well as 30 minutes reperfusion in the

PostFTY group). This increase in PKB/Akt activation might explain the increase in GSK-3β phosphorylation observed at this stage, or it could be that FTY720 induces the phosphorylation of GSK-3β via PKA, since PKA has been implicated in the signalling pathways activated by sphingosine (Vessey *et al.*, 2008). In the setting which we investigated, ERK p42/p44 activation was unaffected by PP2A, since it remained phosphorylated during early reperfusion.

If this model is true, then the final effects of FTY720 on signalling, and obviously also on the eventual actions elicited by these signalling pathways, are determined by the balance between these different pathways; as this balance shifts over time, the effects of FTY720 shifts concurrently. This model therefore also contributes to our understanding of how FTY720 can exert different effects on IFS and functional recovery and why its effects are dose dependent.

The question however arises why we did not see such a robust signalling response to reperfusion administered FTY720, as with pre-ischaemic FTY720 treatment. There are two possible reasons for this, both related to the fact that reperfusion itself also phosphorylates these signalling kinases: (1.) It could be that PKB/Akt and p38 MAPK are already nearly maximally phosphorylated at the time point we investigated due to reperfusion itself; or (2.) FTY720 and reperfusion possibly stimulate the activation of PKB/Akt and p38 MAPK through common pathways, which become saturated under these conditions and therefore unable to induce further phosphorylation. Irrespective of the mechanism, at 15 minutes reperfusion FTY720 is unable to stimulate further phosphorylation of PKB/Akt and p38MAPK. Reperfusion possibly masks the stimulatory effects of FTY720 at this time point.

Contextualizing our data with regards to PP2A in I/R, FTY720 exposed PKB/Akt, GSK-3β and p38 MAPK as potential substrates for PP2A, especially during ischaemia and the very first minutes of reperfusion.

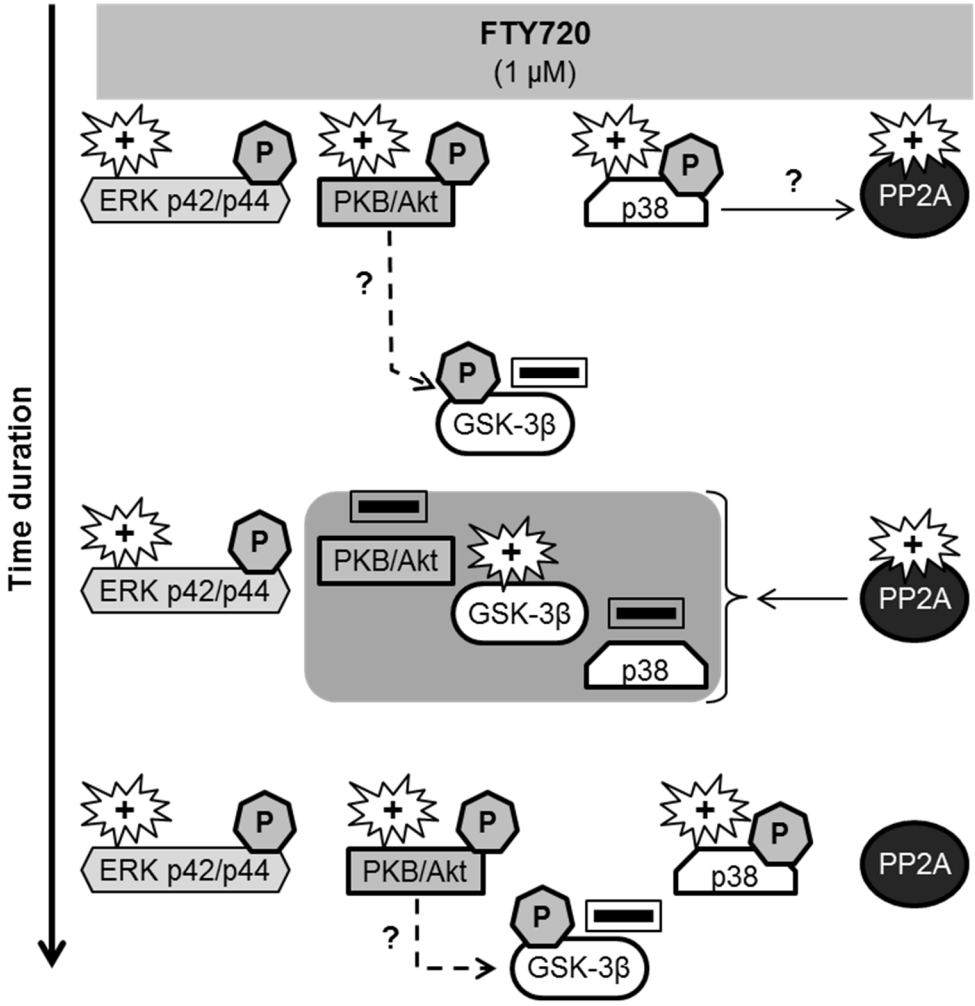
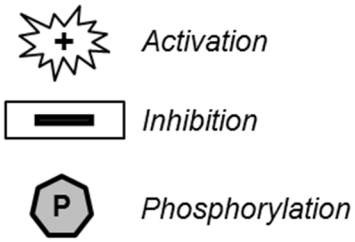


Figure 5.60: Proposed effect of FTY720 on intracellular signalling.
FTY720 activates several signalling pathways, with the final effects being determined by the balance between these pathways. In this model FTY720 activates PP2A, which in turn targets and dephosphorylates PKB/Akt, GSK-3β and p38 MAPK. These effects are however very transient, and as soon as PP2A activity dissipates, FTY720 and/or I/R stimulated activation of these kinases dominate again.
Legend:



The signalling-based explanation for the effects of FTY720 on IFS

Having established the kinase profiles associated with FTY720 administration, an explanation involving these pathways could also be put forward for the effects of FTY720 on IFS. We found that pre-ischaemic treatment with FTY720 (1 µM) increased IFS. This could be due to the reduced phosphorylation of PKB/Akt and the activation of GSK-3β at the very onset of reperfusion. As noted in Chapter 1, the very first minutes of reperfusion is important in determining the eventual outcome of I/R injury.

Reperfusion treatment with FTY720 exerted an infarct sparing effect. Since samples for this group were collected at 15 minutes reperfusion only, possible effects of FTY720 on PKB/Akt and ERK p42/p44 signalling in the first moments of reperfusion could have been missed. It is however entirely conceivable that FTY720 induced a pronounced increase in the activity of these pro-survival kinases in the first moments of reperfusion, thereby mediating cardioprotection.

FTY720 in the isolated rat heart exposed to I/R: limitations and future directions

This study serves as a good basic and general description of the effects of FTY720 in the setting of myocardial I/R. There are especially three novel aspects to this study: (1.) the inclusion of a pre-ischaemic FTY720 administration group; (2.) investigation and contextualization of the effects of FTY720 on PP2A; and (3.) the description of the effects of FTY720 on GSK-3β and p38 MAPK. The well-known pro-survival signalling kinases, ERK p42/p44 and especially PKB/Akt have already received much attention with regards to S1P and FTY720 signalling.

We were however confronted with several unknown factors which complicated the understanding and interpretation of our results:

1.) The actual level of PP2A activity. Although we are confident that FTY720 at the dose we used activates PP2A, we are not sure how long this effect persists and if it is maintained in the context of a cellular environment which favours the inhibition of PP2A – i.e. at the end of ischaemia and the onset of reperfusion (see Chapter 3). We used PP2A phosphorylation as a read-out of PP2A activity, although it is not the only factor which determines its activity (see the introduction to Chapter 3). It would therefore be worthwhile to assess PP2A activity directly throughout the perfusion protocol.
2.) Following on the previous point, cancer-related research has shown that FTY720 activates PP2A, not necessarily by directly influencing its phosphorylation, but by interacting with one of its endogenous inhibitors, namely SET. To our knowledge not much is known regarding the contribution and importance of SET to PP2A activity in the heart. It would therefore be insightful to characterize SET in this setting as well, specifically with regards to its expression, phosphorylation and interaction with PP2A.
3.) Since FTY720 can act as either a sphingosine or a S1P mimetic, one is not sure whether the effects seen are due to FTY720, or P-FTY720. Future work on this topic will benefit from better characterization of the actions of FTY720. This can be done by the administration of FTY720 in conjunction with SK2 inhibition (pharmacological inhibitor, or even silencing RNA-mediated) in order to block the potential phosphorylation of FTY720, or administration of specifically P-

FTY720 or an unphosphorylatable form of FTY720 (some studies have reported the use of such analogues).

4.) FTY720 was found to elicit very short term effects on signalling. It would be interesting to compare our results with an experimental design aimed at enhancing FTY720 activity. This can be achieved by either increasing the concentration of FTY720 used, or lengthening the duration of FTY720 administration. Due to concentration dependent effects of FTY720, the former option may not be desirable. Increasing the length of administration may be more feasible. Others have used much longer FTY720 administration periods than we have, for example Vessey *et al*. (2013) administered FTY720 for 40 minutes of reperfusion, or Egom and colleagues (2010) who administered FTY720 to their isolated perfused rat heart model for a total period of 50 minutes.

5.) We observed surprisingly little effects of reperfusion administered FTY720 on the signalling kinases, despite the fact that this protocol elicited an infarct sparing effect. As already mentioned in the discussion this is probably because we only investigated the kinase profiles at the end of the FTY720 perfusion period (15 minutes), and then again at the end of the experiment (35 minutes). It would be informative to include more time points for kinase profiling; specifically at the onset of reperfusion, as well as between the administration of FTY720 and the end of the experiment.

6.) As already mentioned, the precise effects of FTY720 in these signalling cascades are difficult to assess within the already complex setting of I/R. Although this is the scenario in which we are interested, it would also be interesting to assess the activity of these kinases during, as well as after FTY720 administration, under normal baseline conditions. This would shed light on FTY720 initiated signalling *per se*.

7.) The latter point can also be extended to include measuring the effects of FTY720 administration on function under baseline conditions, by either administering FTY720 during work mode perfusion or administering FTY720 to a non-recirculating retrograde perfusion model with a balloon inserted into the left ventricle to assess function throughout. This approach would be of value since, although we speculate that the negative effects of FTY720 on reperfusion function could be due to its negative inotropic and bradycardiac effects, we never determined the direct effects of FTY720 administration on cardiac function in our model.

It is clear that much research still needs to be done on this interesting and potentially useful drug.

FTY720 in the isolated rat heart exposed to I/R: summary and conclusion

We found that FTY720 administered to the isolated rat heart exposed to I/R exerted divergent effects which were dependent on the dose administered. In general, FTY720 exerted an infarct sparing effect, which was not associated with improved functional recovery. In fact, 2.5 µM FTY720

suppressed functional recovery. Pretreatment with 1 μM FTY720 also augmented IFS. These disparate effects are probably due to the fact that FTY720 can either directly exert an effect on the cardiovacular tissue, or it can be phosphorylated and then signal through the S1P signalling machinery. Some of the possible mechanisms by which FTY720 could have exerted its beneficial and detrimental effects in our model include: elevation of S1P levels and S1P associated signalling, activation of sphingosine mediated signalling and negative inotropic and bradycardiac effects on the heart. Our results caution against the potential clinical application of FTY720 in the setting of myocardial I/R injury, since at different concentrations it can elicit a wide variety of effects.

Concerning FTY720 as a tool to activate PP2A, we found that this activation of PP2A occurred within the context of simultaneous co-activation of several intracellular signalling pathways and proved to be very transient. This observation that FTY720 simultaneously activates kinase- and phosphatase mediated pathways implies that one should be cautious to relate the effects elicited by FTY720 with PP2A activation alone. Keeping this in mind, our data places PP2A as a phosphatase with the potential to dephosphorylate GSK-3β and p38 MAPK during sustained ischaemia, as well as PKB/Akt within the first minutes of reperfusion. Activation of PP2A at the onset of reperfusion could however therefore be detrimental, since the associated inactivation of PKB/Akt and activation of GSK-3β is associated with an increase in IFS in our model.

FTY720 therefore clearly identified PP2A as a potentially important protein phosphatase at the junction between sustained ischaemia and early reperfusion, with the potential to target the PKB/Akt – GSK-3β pathway, as well as p38 MAPK.

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